THE AMERICAN JOURNAL OF PATHOLOGY

VOLUME XXXIX NOVEMBER, 1961 NUMBER 5

A QUANTITATIVE STUDY OF CORONARY ARTERIAL CALCIFICATION

LARRY E. BOLICK * AND DAVID H. BLANKENHORN, M.D.†

From the University of Southern California School of Medicine, Los Angeles, Calif.

Calcification is generally considered to occur at a late stage in the formation of atheromas and may represent an irreversible event in this process. Much remains unknown concerning the manner in which calcium accumulates. It has been difficult to study the structural pattern of calcification in advanced atheromas because these lesions must be decalcified before they can be properly sectioned. In the present study, coronary atheromas with and without gross evidence of calcium deposit and segments of normal coronary artery have been extracted with ethylenediamine tetra-acetic acid (EDTA) by a new method which permits determination of calcium content, determination of calcium removal rate, and microscopic examination of the decalcified specimen. This approach has yielded new information regarding the distribution of atheromatous calcium and has shown calcium deposition to be related to hematoxylin-ringed lacunar spaces, a morphologic feature of atheromas not previously associated with mineral deposits.

MATERIAL AND METHODS

Coronary atheromas and segments of normal coronary arteries, obtained at necropsy, were fixed in 25 volumes of 4 per cent aqueous calcium-free formalin for periods of 3 to 15 days. All solutions used in this experiment were made with water which was distilled once, and then passed through an ion exchange column.‡ Care

Supported by United States Public Health Service Grant #4699 and the Los Angeles County Attending Staff Association, Project #1020.

Accepted for publication, June 15, 1961.

* This work was done during tenure of a summer student research fellowship supported by Boyle & Company, Pharmaceuticals. Present address: Madigan Army Hospital, Tacoma, Wash.

† Associate Professor of Medicine, Department of Medicine.

‡ The Universal Model Deionizer, Illinois Water Treatment Co., Rockford, Ill.

was taken to prevent tissues from drying before fixation. After fixation, specimens were blotted dry on filter paper, weighed, and transferred to individual polyethylene bottles containing known volumes of 0.34 M NH_4-EDTA (100 cc. per gm. of tissue). These were kept in a 5° C. cold room and gently agitated for 14 days. Following this extraction, specimens were dehydrated in graded alcohols, embedded in paraffin, sectioned, and stained with Harris' hematoxylin and eosin.

NH_4-EDTA (0.34 M) was prepared from NA_2-EDTA * as follows: 500 gm. of NA_2EDTA were dissolved in 5 l. of water and concentrated hydrochloric acid (HCl; approximately 300 ml.) was added to bring the solution to pH 1.5. EDTA was allowed to precipitate for 12 hours, separated by decantation, and washed with 2 volumes of water. Next, the precipitate was dissolved in 4 l. of water and the pH adjusted to 7 with saturated ammonium hydroxide. EDTA was again precipitated in its acid form with concentrated HCl, washed, dissolved in water, adjusted to pH 7 with ammonium hydroxide, and stored in polyethylene containers. NH_4-EDTA prepared in this manner leaves no ash when heated to 500° C. and allows quantitative recovery of as little as 50 μg. of calcium from 0.9 gm. of NH_4-EDTA.

At frequent intervals during the period of extraction, aliquots of EDTA solution were withdrawn for calcium assay. One to 5 cc. samples of the solution were transferred to test tubes (Kimble #45048, 19 by 150 mm., Kimax), and dried at 105° C. Test tubes were next heated to 500° C. in a muffle oven for 8 hours. The ash was dissolved in 4 to 6 drops of 15 per cent aqueous perchloric acid and reheated to 200° C. for an hour. Residual ash was dissolved in 1 ml. of 0.1 N HCl, and calcium determined by titration as follows: 2 ml. of 1.25 N potassium hydroxide (stored in a polyethylene container), 1 drop of 1 per cent sodium cyanide, and 0.4 cc. of 1 per cent Cal-Red were added.† Titration was performed with 2.6 M NA_2-EDTA, standardized against calcium carbonate and delivered from a microburette. This determination is specific for calcium. Magnesium is not measured by EDTA titration when Cal-Red is used as the indicator. Interference by copper, aluminum and iron is eliminated by the addition of potassium hydroxide and sodium cyanide.

From these determinations, calcium content of the atheroma at the beginning of extraction and at the time of each sampling was calculated. The rate of calcium extraction from tissue by chelating solution was then determined by solving the equation: $K = \frac{2.303}{t_2 - t_1} \times \log \frac{C_1}{C_2}$. ($K$ = extraction rate; C_1 = calcium content at t_1; C_2 = calcium content at t_2; t = time in hours.)

Results

Table I gives the calcium content of normal coronary artery and coronary atheromas. Complete removal of calcium by NH_4-EDTA was demonstrated in 3 of these plaques by ashing them and finding no residual calcium. Calcium content is given in per cent of wet weight to allow comparison with previous data for aortic and iliac lesions.[1-3] When coronary atheromas show gross evidence of calcification, they may contain as much or more calcium based on percentage of wet weight as advanced aortic or iliac lesions.

Text-figure 1 illustrates the extraction rates of 8 atheromas. The loga-

* Geigy Chemical Corporation, Saw Mill River Road, Ardsley, N.Y.
† Scientific Service Laboratory, Inc., P.O. Box 175, Dallas 21, Texas.

rithm of the calcium content of lesions during EDTA extraction is plotted against time, and a straight line results in all instances, indicating an essentially constant rate of calcium removal. This finding allows

TABLE I

CALCIUM CONTENT OF NORMAL CORONARY ARTERIES AND CORONARY ATHEROMAS

Case No.	Sex & age (yr.)	Cause of death*	Specimen	Calcium, per cent of wet weight
65302	M 70	Mesenteric thrombosis	1. Atheroma + Ca†	11.3
			2. Atheroma + Ca	14.1
			3. Atheroma — Ca	1.08
65428	M 68	Gastric carcinoma with metastasis	5. Normal coronary artery	< 0.09
			6. Atheroma + Ca	10.1
65390	M 66	Esophageal carcinoma with metastasis	7. Atheroma + Ca	2.42
65421	M 65	Arteriosclerotic heart disease; congestive failure	8. Atheroma + Ca	10.3
			9. Atheroma + Ca	10.3
			12. Atheroma + Ca	8.6
65474	M 80	Arteriosclerotic heart disease; massive intracerebral hemorrhage	10. Atheroma + Ca	14.1
			11. Atheroma + Ca	13.7
			13. Atheroma + Ca	2.08
			14. Atheroma + Ca	13.9
			16. Atheroma + Ca	12.6
65399	F 81	Carcinoma of pancreas with metastasis	17. Atheroma — Ca	0.69
			18. Atheroma + Ca	5.26
65426	F 81	Acute necrotizing pancreatitis	19. Normal cornonary artery	< 0.04
			20. Normal coronary artery	< 0.04
65427	F 85	Arteriosclerotic heart disease; congestive failure	21. Normal coronary artery	< 0.04
			4. Normal coronary artery	< 0.04
			15. Atheroma — Ca	< 0.07

* Determined at necropsy.

† + Ca = gross calcification; — Ca = no gross calcification.

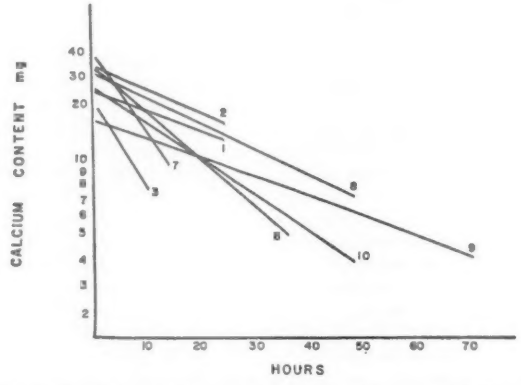
calcium exchange rates to be calculated by the formula given, which describes the rate of a unimolecular chemical reaction.[4]

Heavy, blue hematoxylin staining (Figs. 2 and 3) was frequently encountered in these atheromas although all calcium had been removed. This confirmed the work of Cameron[5] who previously reported that hematoxylin staining might take place in calcific lesions after complete removal of calcium. The extent and intensity of hematoxylin staining could not be correlated with total calcium content or extraction rates. Lack of correlation between hematoxylin staining and calcium content of aortic atheromas has been reported previously.[6]

During a search for morphologic features which might be correlated with calcium contents and extraction rates, the appearance of connective tissue in a plaque with high calcium content and slow extraction rate attracted attention because it was riddled with small, irregular, hematoxylin-ringed lacunas (Figs. 1 and 4). These lacunas had an appear-

ance somewhat suggestive of capillaries or lymphatic spaces and were so numerous that they comprised a considerable portion of the lesion. However, the plaque was not vascular, was quite hard upon gross examination, and contained 10 per cent calcium (wet weight).

Serial sections from all plaques whose extraction rates are illustrated in Text-figure 1 were searched for lacunas. These were easily recognized, and two examiners classifying each plaque independently were in complete agreement as to whether a plaque could be classified as containing lacunas or not. The results are shown in Table II. The rate of calcium removal in plaques containing lacunas was significantly slower than those which showed none.



TEXT-FIGURE 1. The rate of calcium removal from atheromas. Each line demonstrates a change in calcium content in one plaque, identified by the number assigned to it in Table I. The calcium content of plaques 3 and 7 has been multiplied by 10.

DISCUSSION

The procedure employed in this experiment is new and provides a unique method for the study of tissue calcification because both the calcium content and calcium extraction rate of tissues examined microscopically can be known. Previous combined chemical and histologic studies of vascular calcification have employed methods for calcium assay which required destruction of the tissue; thus, microscopic examination of the tissue assayed could not be done. The present technique is made possible by the use of a new salt of EDTA which is readily destroyed by heat, leaving an ash-free residue. EDTA cannot be purchased in the form required, but disodium EDTA is readily converted to the proper salt by a technique described here. The staining characteristics of tissue decalcified by this new method are comparable to tissue decalcified by other techniques, and calcium removal is rapid and complete.

Serial determinations of the calcium content in extraction solutions

have demonstrated that extraction of atheromatous calcium by NH_4-EDTA follows a definite pattern. It has been shown that the calcium content of plaques declines in an exponential manner at a rate which is

TABLE II

CALCIUM EXTRACTION RATE

Case No.	Plaque*	Lacunas		Average extraction rate (μg./hr.)
65302	1	Yes		26.1
65302	2	Yes		29.2
65421	8	Yes		31.7
65421	12	Yes		24.3
			Average	27.8
65302	3	No		78.1
65428	6	No		60.3
65390	7	No		82.2
65474	10	No		48.5
			Average	67.2
				$t = 4.38$†
				$p < .01$†

* The numbers are specimen numbers from Table I.

† The t and p values were determined by standard statistical techniques.[7]

constant and unique for each plaque. Exponential equations have been solved to determine the extraction rate for each plaque shown in Text-figure 1 and these plaques can be divided into two classes: slowly extractable plaques and rapidly extractable plaques. Difference in extraction rate cannot be attributed to factors introduced by the patient's age, sex, or general metabolic state because both rapidly and slowly extractable plaques have been found in the same individual. In addition, because both rapid and slow rates have been found in heavily calcified atheromas, different extraction rates cannot be attributed to differences in the extent of calcification. It seems more probable that extraction rates are determined by the distribution of calcium in atheromas.

It has been shown that extremely hard, heavily calcified lesions may be riddled with lacunar spaces when all calcium has been removed. Clefts are known to occur in lipid-rich atheromas after extraction with fat solvents, and so it seems reasonable to believe that spaces may be left in heavily calcified atheromas when calcium is removed. The possibility that decalcification may leave spaces in tissue does not appear to have received previous consideration. At present, because heavily calcified portions of atheromas cannot be properly sectioned prior to removal of calcium, evidence that the removal of calcium can cause such spaces remains indirect.

The lacunas described in this paper are not unique to atheromas

treated with NH_4-EDTA; they can also be found in plaques decalcified with NA_2-EDTA or with mineral acids. It is possible that their size and number depend upon completeness of calcium removal. They may be more numerous in the present series of atheromas than in material decalcified by other means. Because NH_4-EDTA extraction is the only present method allowing the same tissue to be examined microscopically and assayed for calcium, this question cannot be answered with certainty.

Extraction of calcium is significantly slower from atheromas which are left with lacunas than those which are not. If lacunas are considered to be the sites of previous discrete masses of calcium, this difference in rate can be explained as follows: Calcium contained in granules large enough to leave visible spaces must be assumed to be in larger deposits than calcium in a form too diffuse to leave visible spaces. Geometric considerations indicate that larger masses have a smaller ratio of surface to mass and therefore can be expected to be extracted more slowly. It therefore seems probable that two different forms of calcific deposits occur in advanced atheromas—a diffuse and a discrete form. The diffuse form is rapidly extractable and leaves no visible spaces. The discrete form is slowly extractable and leaves visible spaces. It should be emphasized that although extraction rate data and the occurrence of lacunas are reported together and correlated, they represent two independent lines of evidence, and both suggest that calcification in advanced atheromas can occur in more than one form.

Conclusions

A quantitative method of decalcifying tissues which utilizes NH_4-EDTA is described. Total calcium content of coronary vessels and calcium removal rates have been determined upon specimens left intact for microscopic examination. This technique indicates that coronary atheromatous calcification can be as extensive as that which occurs in aortic atherosclerosis. In addition, the method gives evidence that two different forms of calcification occur in coronary atheromas. Certain calcific atheromas exhibit characteristic hematoxylin-ringed lacunas when calcium is removed. These atheromas are slowly extracted by EDTA and are believed to contain calcium in discrete granules. Other calcific atheromas show no lacunas and are rapidly extracted by EDTA. These atheromas are believed to be more diffusely infiltrated by calcium.

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[*Illustrations follow*]

Legends for Figures

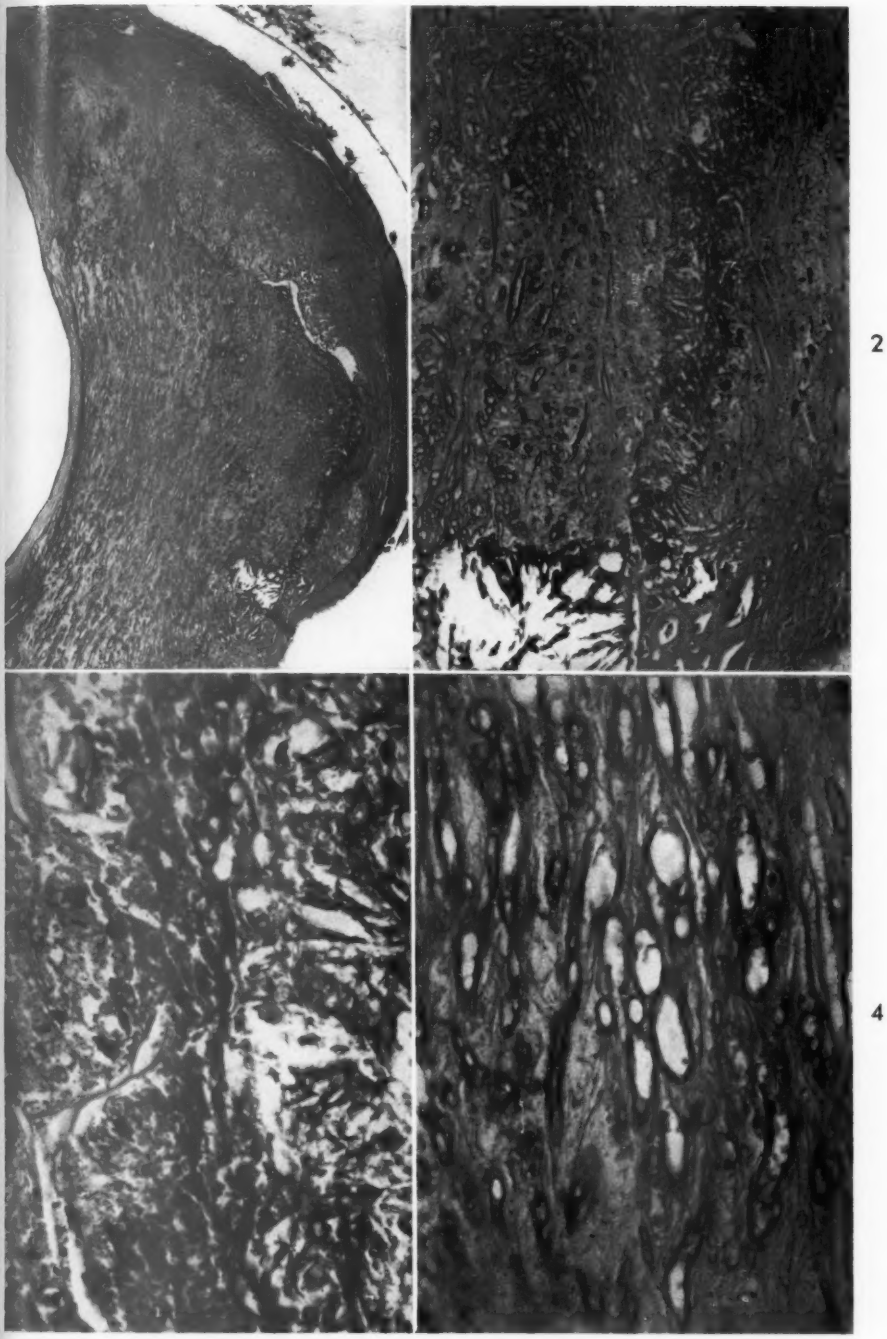
Photomicrographs were prepared from sections stained with hematoxylin and eosin. They show microscopic detail of a very hard, heavily calcified atheroma. Ten per cent of the weight of this plaque was calcium.

Fig. 1. The plaque in eccentric. Heavy hematoxylin staining occurs in two areas, but the major portion of the plaque is filled with lacunar spaces. × 30.

Fig. 2. Detail from the outer margin of the plaque, showing heavy hematoxylin staining adjacent to an area containing lacunar spaces. × 125.

Fig. 3. Detail of the heavy hematoxylin-stained granules shown in Figure 2. × 500.

Fig. 4. Lacunar spaces from the center of the lesion. Hematoxylin stains their margins. × 500.



THE DISTRIBUTION OF FIBRIN WITHIN THE AORTIC INTIMA

An Immunohistochemical Study

Neville Woolf, Ph.D., M. Med.

From the Department of Pathology, St. George's Hospital Medical School, (University of London), London, England

The introduction by Coons and his associates[1-3] of the fluorescent antibody technique has made possible the identification in tissues of a wide range of substances both native and foreign. The scope of morphologic studies has thus been greatly extended by this addition to the tissue pathologist's armamentarium. When conventional histologic methods are used, fibrin in tissue sections gives markedly variable results, and this variability is seen to obtain even when fibrin clots, formed *in vitro*, are sectioned and treated by the usual histologic means for the identification of this protein.[4] Negative reactions for fibrin in sections, when these conventional procedures are employed, may be entirely without validity, and no adequate evaluation of its presence can be made unless a sensitive method based on immunologic specificity is utilized.

This is of particular importance in relation to the pathogenesis of atherosclerosis. Few morphologists would now deny that recurrent deposition of fibrin on the arterial wall and its subsequent incorporation play an important part in the growth of the mature atherosclerotic plaque.[5-11] It is still, however, an open question as to whether fibrin deposition either on or within the vessel wall has any role in the earlier stages of atherogenesis, and whether it contributes in any way to the diffuse intimal thickening of the vessel wall which is an inevitable accompaniment of the aging process.

The present study reports the application of Coons's fluorescent antibody technique to an investigation of the presence and distribution of fibrin in macroscopically normal areas of the aorta in various age groups, in early atherosclerotic lesions such as gelatinous elevations and fatty streaks, and in mature atherosclerotic lesions. It extends a previous study which was limited to the examination of fatty streaks.[12]

The material on which this paper is based formed part of a thesis submitted for the degree of Ph.D. (London).

The work was carried out during the tenure of a William Shepherd Research Fellowship in St. George's Hospital Medical School, Department of Pathology, made available by the Governors of St. George's Hospital, London.

Accepted for publication, June 15, 1961.

Material and Methods

Tissue blocks from the aortas of 115 subjects were used. These ranged in age from newly born infants to adults of 70 years. The blocks of tissue, in which no intimal lesions could be detected with the naked eye, were selected in all cases from immediately above the renal artery ostiums so as to minimize as far as possible the effects of the known differences in intimal thickening in various parts of the aorta.

All types of atherosclerotic lesions were selected for sectioning, with the exception of those in which a marked degree of calcification was present. Decalcification was avoided in an attempt to prevent denaturation and possible alteration in the immunologic reactivity of the tissue proteins. In view of the comparative ease with which fibrin can be demonstrated in advanced atherosclerotic lesions by conventional histologic methods, the bulk of the lesions studied were fatty streaks and small lipid plaques not exceeding 4 mm. in diameter.

Small blocks of fresh tissue were quenched at −70° C. in a mixture of acetone and carbon dioxide snow and stored at −20° C. until required. Sections were cut 7 to 10 μ thick in a modified Linderstrøm-Lang cryostat,[8,13] running at −21° C., were mounted in the cold and then dried in a current of cool air.

Before treatment with coupled antiserums, the sections were washed in 4 changes of 0.15 M. of sodium chloride at room temperature in order to dissolve out soluble fibrinogen in the tissues.[14]

Fluorescein-coupled serums were then carefully layered onto the sections and these were kept in a moist atmosphere at room temperature for 90 minutes. The serums were then drained off and the sections washed in 3 changes of buffered saline before being mounted in buffered glycerol (pH 7).

Throughout the investigation, a rabbit anti-human-fibrin serum coupled with fluorescein isothiocyanate[15] was used. The preparation and characterization of this serum has been described in a previous communication.[16] The specificity of the immunohistochemical procedure at the slide level was controlled by treating sections adjacent to that to be tested in the following ways:

1. By treatment with an aliquot of fluorescein-coupled anti-fibrin previously adsorbed with washed, finely ground, human fibrin in excess.
2. By immunologic blocking—pretreatment of a section with an unconjugated aliquot of the anti-fibrin serum before exposure to the fluorescein-coupled anti-fibrin serum (Figs. 7 and 8).
3. A fluorescein-coupled serum from a nonimmunized rabbit was also used to exclude the possibility of nonspecific staining.[17]

Results

In all the sections, the elastic fibers of the media were brilliantly autofluorescent, but the silver-blue fluorescence characteristic of elastic tissue was easily distinguishable from the bright yellow-green of fluorescein. Specific yellow-green fluorescence was absent from all the control sections, indicating that the fluorescein staining seen in the test sections was due to a specific immunologic reaction at the slide level.

The Macroscopically Normal Aorta in Infants and Children

Sections of aorta from infants and young children showed differences in intimal thickness, not only between one case and another but between adjacent blocks from the same aorta. In the newborn and in infants a few months old, the intima in most cases was extremely thin, only a few

strands of tissue separating the usually well-marked internal elastic lamina from the lumen. Under ultra-violet light this intimal tissue gave a very dull bluish autofluorescence against which the brilliant silver-blue color of the internal elastic lamina stood out boldly. Focal splitting of the internal elastic lamina marking the beginning of the formation of the musculo-elastic layer was clearly seen in some sections. When these sections were treated with fluorescein-coupled anti-human-fibrin serum, a line of brilliant specific fluorescence was uniformly noted along the lumen surface of the vessel wall (Fig. 1).

At first it was thought that this might be due to nonspecific staining of endothelial cells.[18] Pretreatment of adjacent sections with unconjugated anti-fibrin serum blocked subsequent fluorescein staining, and the other controls gave similarly negative results. The degree of optical resolution obtainable with sections of such thickness was not such as to enable one to state whether the thin superficial layer of fluorescent material lay on the lumen surface of the endothelium or not. It is possible that this fluorescence might result from the deposition on the vessel wall of a fine layer of postmortem clot. With death of the endothelial cells, small amounts of tissue thromboplastin might be released locally and so initiate such clotting. In addition to the uniform presence in these sections of a superficial thin layer of fluorescent material, most of the sections of aortas from infants and children also showed a few flecks of specific fluorescence situated more deeply within the intima.

When sections of aorta in infants and children were taken through or adjacent to points of branching, very different appearances were observed. Here, disruptive changes in the internal elastic lamina were marked and the intima was greatly thickened. When treated with coupled anti-human-fibrin serum, the thickened intima showed the presence of far more fluorescent material than could be demonstrated in sections from the same specimen selected from areas a centimeter away from the point of branching (Fig. 2). The specific fluorescence was diffusely distributed throughout the localized intimal thickening. Adjacent sections from these blocks sometimes showed the presence of a few droplets of sudanophilic material in the thickened intima. This was usually trifling in amount and was certainly not an invariable constituent of these intimal cushions.

The Macroscopically Normal Aorta in Older Age Groups

These sections came from subjects ranging in age from 17 to 36 years, and in most cases the intima was fairly thick. Despite the fact that these tissue blocks had been selected from areas which had appeared normal on gross examination, some of the sections showed a considerable degree

of edema, with separation of formed connective tissue elements. The intima in these areas was usually the site of an accumulation of connective tissue mucopolysaccharide, and appropriately stained sections showed the presence of finely dispersed, apparently extracellular, sudanophilic material. These histologic appearances cannot be regarded as being completely normal and correspond more closely with those described in focal gelatinous elevations. After treatment with the fluorescein-conjugated anti-fibrin serum, the intima in these sections showed a large amount of specific fluorescence (Fig. 3). No fluorescein staining was noted in the media. The fluorescent material was distributed fairly diffusely throughout the affected intima in the form of irregular flecks, occasional coarser aggregates and fine threads which usually lay parallel to the lumen edge of the intima. The fluorescence was most marked in the superficial levels of the intima, but this quantitative difference was not striking.

Early Atheromatous Lesions in the Aorta

The early fatty lesions studied fell into two groups. First were those in which intimal elevation was barely perceptible to the naked eye. When frozen sections of such lesions were stained with a mixture of Sudan III and IV, the stainable fat was seen to be present both in intra- and extracellular forms. This fat had not yet been swept up and packed into a single aggregate of foamy macrophages lying immediately under the endothelium.

Sections through lesions of this type, when treated with the dye-coupled anti-fibrin serum, showed the presence of abundant specific fluorescent material throughout the thickened area of the intima. There was a marked increase in the amount of intimal fluorescence noted here, as compared with the amount noted in sections from macroscopically normal areas of the aorta. The fluorescent material was distributed throughout the intima, again in the form of small irregular aggregates and short fine threads. In some cases the most brilliant fluorescence was seen in those areas where sudanophilic droplets were present.

The second group of lesions examined were those which, while no larger in area than those just described, were more markedly elevated above the surface of the surrounding intima. Within these localized intimal elevations the stainable fat was almost entirely intracellular and was gathered together to form a tightly packed mass of foamy macrophages.

The preparation and treatment of frozen sections of such lesions presented certain technical difficulties which were never entirely overcome. During repeated saline washings, the substance of these miniature plaques tended to disintegrate and the lipid-laden macrophages were

washed out of the sections. As a consequence, the endothelial lining and those tissues lying immediately beneath the endothelium tended to lift, thus exaggerating the degree of intimal elevation actually present.

Despite this loss of substance, these localized intimal elevations exhibited brilliant fluorescence when treated with the coupled anti-fibrin serum, and in these sections it was possible to see that the layers of specific fluorescent material were lying in the form of long threads immediately beneath the endothelium (Fig. 4).

In slightly more advanced lesions where the stainable fat tended to be separated from the lumen by a few fibrous strands, the appearances were less uniform. Where only a very thin layer of fibrous tissue lay between an aggregation of fat-filled cells and the vessel lumen, a few long strands of fluorescent material were seen lying beneath the endothelium and roofing over the area where the lipophages had been. As the lesions became larger, a tendency was noted for the fluorescent material to appear less as a diffuse stippling of the intima than in the form of definite threads and coarse aggregates. In some instances the threads lay parallel to the endothelium; in others they were arranged to form a coarse network in the interstices of which lipophages could be seen.

Advanced Atherosclerotic Lesions in the Aorta

The reasons for examining lesions of this type were somewhat different from those relating to the preceding sections. In mature atherosclerotic plaques, it is not difficult to demonstrate the presence of fibrin by conventional histologic means. The object of applying the immunohistochemical technique to these mature lesions was primarily to observe how closely the distribution of specific fluorescence mirrored the distribution of fibrin, as indicated by Mallory's phosphotungstic acid-hematoxylin (PTAH) method, and secondarily to compare the amounts of material in a section which would react positively with both methods. Both of these objectives were attained in that where fibrin could be demonstrated by histologic means in paraffin section, frozen section of half of the same tissue block showed the presence of brilliant specific fluorescence in more or less the same sites. In the main, the use of conjugated anti-fibrin serum showed more fibrin to be present within the tissue section than did the conventional staining methods. Within the substance of a fully developed atheromatous plaque, massive accumulations of fluorescent material were often present, but a striking feature was the fact that this material was not diffusely distributed and between two large masses of fluorescent material there were areas of the intima in which very little of this substance was found (Fig. 5). In plaques where there had been breakdown of much of the substance, with a subsequent increase in the mobility of the intima relative to the under-

lying media, the presence of material reacting positively both with the coupled anti-fibrin serum and Mallory's PTAH stain was noted in the angle between the hyalinized and thickened intima and the media. According to Duguid and Robertson,[19] small hemorrhages frequently take place at this site, and the finding of fibrin in these plaque angles constitutes supportive evidence for their view.

Discussion

The evaluation of the experimental data presented above falls naturally into 3 parts. Before there can be any speculation as to the possible relevance of these findings to atherogenesis in the human subject, it is necessary to be sure, firstly that the immunohistochemical method used here has demonstrated the presence of fibrin within the sections and, given this, that the presence of this protein represents an antemortem phenomenon.

In view of the negative reactions obtained with the various control procedures and the fact that the antiserum contains a single precipitating antibody directed against human fibrin, it seems not unreasonable to assume that the brilliantly fluorescent material seen in the intima of the human aorta is fibrin.

Florey[20] has suggested that the presence of fibrin in aortic fatty streaks, as demonstrated by the immunohistochemical technique, may represent postmortem insudation of plasma resulting from an alteration in endothelial permeability taking place after death. In the absence of a pulse pressure to initiate and maintain plasma infiltration, it seems unlikely that plasma fibrinogen could penetrate as deeply into the depths of the intima as has been seen in this series of experiments. Moreover, prolongation of the postmortem interval with an increased time during which infiltration could take place has not shown an increase in the amount of fibrin visualized in the intima by this technique. To investigate this point more fully, tissue blocks from resected coarctations of the aorta were quenched immediately after removal from the patient. Preceding resection, the aorta had been occluded by tapes proximal and distal to the coarctation, and thus no blood passed through the affected portion of aorta immediately before removal. When fatty streaks and plaques from such specimens were examined after having been treated with coupled anti-human-fibrin serum, fibrin was found to be deeply situated within the intima, in some instances separated from the lumen of the vessel by endothelialized mural thrombi (Fig. 6). It seems likely, therefore, that the intimal fibrin deposits arise before death. There is, however, one possible exception to this. In the case of the infant aortas in which a thin superficial layer of fluorescent material was seen ad-

jacent to the lumen, it was impossible, because of the thickness of the sections, to ascertain whether the fibrin lay on the lumen aspect of the endothelium or beneath it. These fine deposits may well represent post-mortem clotting.

There is, at present, no direct means of ascertaining the mechanisms by which these various types of fibrin deposit are mediated and what implications they have, not only in relation to the pathogenesis of atherosclerosis but with regard to the diffuse intimal changes associated with aging. We know from the morbid anatomic observations of Duguid [6] and Crawford and Levene [8] and also from the experimental data of McLetchie,[21] that very thin layers of fibrin lying on the lumen surface of a large vessel can rapidly become covered with endothelium and incorporated within the vessel wall, thus leaving the intima slightly thicker than previously. In my own view there is insufficient evidence to postulate that the thin superficial layer of fibrin uniformly seen in sections of infant aortas is an example of this process in operation. Apart from the serious doubts as to whether this type of deposit is ante mortem in nature, the intimal thickening which takes place during the first decade of life is accompanied by highly specific alterations in the structural pattern of the vessel wall, with particular reference to the formed connective tissue elements. The orderly nature of the changes in elastic tissue and muscle and their uniform occurrence suggest that remodeling and growth of the vessel wall are taking place as a functional adaptation to hemodynamic requirements rather than that these changes represent a reaction to the presence of fibrin within the intima.

In the macroscopically normal aortas of older subjects in whom the aorta has already developed its definitive pattern, a marked increase in the amount of fibrin demonstrable by the immunohistochemical technique was noted. This was diffusely distributed throughout the thickness of the intima, but there was a tendency for the fibrin to be concentrated in the more superficial layers of the intima. Where fibrin persists in any tissue, there is a tendency for it to be replaced ultimately by reticulin and collagen, and the local conditions in the aortic intima may well be such as to encourage the prolonged presence of deposited fibrin. There is no direct capillary blood supply, tissue thromboplastic activity is high, and tissue plasminogen is present in very small amounts. While, however, on theoretic grounds, the combination of these factors and the presence of excess amounts of fibrin within the intima could lead to the formation of fibrous tissue and thus to an increase in thickening of the intima, there are no direct data, as yet, to prove that the accumulation of fibrin within the aortic wall is causally related to diffuse intimal thickening.

In the course of this study it became evident that the distribution of fibrin within the aortic intima conformed in the main to two patterns. In the macroscopically normal aorta and in those fatty streaks where the stainable lipid was distributed diffusely throughout the thickness of the intima, the fibrin was diffusely distributed in the form of fine flecks, fine short threads and tiny stellate aggregates. It seemed difficult here to invoke mural thrombosis and subsequent incorporation of fibrin as being solely responsible for its presence in this form, and it appeared likely that infiltration from the lumen was the operative mechanism. In fatty lesions where the stainable fat is concentrated within a single large agglomeration of lipid-filled macrophages and in more advanced lesions where the lipid-filled cells are separated from the lumen by strands of fibrous tissue, the fibrin appears in the form of long strands and coarse aggregates. The appearance of the fibrin in these lesions suggests that it represents the results of incorporation of a mural deposit on the endothelial surface of the aorta analogous to those described by Duguid [6] and Crawford and Levene.[8]

In the fully developed atherosclerotic plaque, both conventional histologic studies and the results of the application of the fluorescent antibody technique suggest that mural thrombosis with subsequent incorporation of fibrin into the substance of the vessel wall plays the major role in the growth of such lesions. In the present study the fibrin visualized in mature lesions appeared in the form of bulky masses lying at varying levels within the substance of the plaque and tending to lie parallel to the lumen surface. These appearances suggest that each of the major collections of fibrin has been laid down fairly rapidly in the form of a mural deposit and has then been covered by endothelial cells.

If such different mechanisms as infiltration and mural thrombosis with incorporation can account for the presence of fibrin within the aortic intima, then it may be possible to suggest an explanation for some of the anomalies in the natural history and epidemiology of aortic atherosclerosis. The deposition of fibrinogen which has infiltrated the intima may be dependent on such factors as elastic tissue changes at points of stress, with consequent accumulation of connective tissue mucopolysaccharide capable of binding fibrinogen and lipid.[22] The intimal cushion from an infant aorta shown in Figure 2 may be an example of this process. Such intrinsic remodeling and repair processes are probably common to all races and population groups. This may account for the fact that small fatty streaks are as common among children and adolescents from those groups with a low incidence of mature atherosclerotic lesions as in those where the severer forms of atherosclerosis and its sequels are frequent.[23]

If, however, growth of the fatty streak or gelatinous elevation is mediated by a different process—mural deposition of fibrin and the complications which may ensue from it—then it is possible to understand why the natural history of early atheromatous lesions in the two types of population described differ so sharply. This discussion is frankly speculative but does not appear incompatible with much of the observed data relating to aortic atherosclerosis.

Summary

The application of the Coons fluorescent antibody technique to a study of the distribution of fibrin in the aortic intima is described. The amount of fibrin increases with intimal thickness, and the presence of atherosclerotic lesions of various types is associated with a further marked increase. Differing patterns of distribution suggest that infiltration of plasma fibrinogen as well as mural deposition and incorporation may be responsible for the presence of the protein within the intima. Differing emphasis on these mechanisms among different population groups might be partly responsible for epidemiologic differences.

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I am deeply indebted to Professor T. Crawford, head of the Pathology Department, St. George's Hospital Medical School, for his constant encouragement and stimulation. I am also grateful to Dr. L. O. Butler and Professor S. D. Elek for assistance with immunologic aspects of the problem.

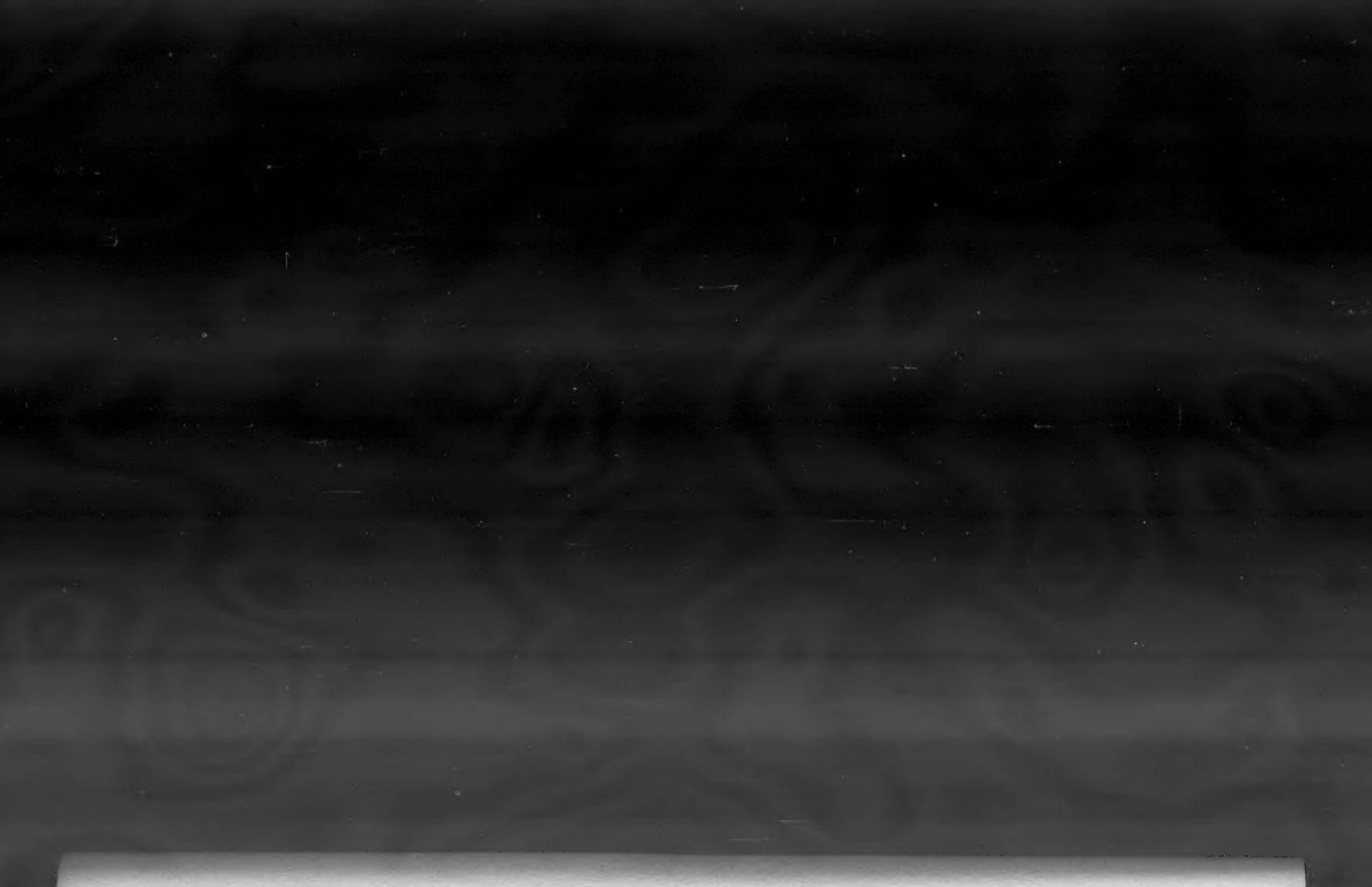
Legends for Figures

Photomicrographs were prepared with ultraviolet light microscopy. Except where indicated, sections were treated with fluorescein-coupled anti-fibrin serum.

Fig. 1. Frozen section of aorta; infant of two months. The elastic fibers of the media and the internal elastic lamina in particular are brilliantly autofluorescent. On the lumen aspect of the rather thin intima is a line of specific fluorescein fluorescence which varies slightly in thickness over different parts of the surface. × 175.

Fig. 2. Frozen section through point of aortic branching; same case as Figure 1. In contrast to the previous section, the intima is markedly thickened and the conspicuous internal elastic lamina cannot be seen. The intima contains a moderately large amount of fluorescent material. × 175.

Fig. 3. Frozen section through grossly normal area of aorta; woman aged 22. Despite its normal appearance on naked-eye examination, this section showed an increase in connective tissue acid mucopolysaccharide and some fine stippling with sudanophilic material. Treatment with the anti-fibrin serum shows finely dispersed particles of fluorescent material diffusely distributed throughout the intima. × 175.



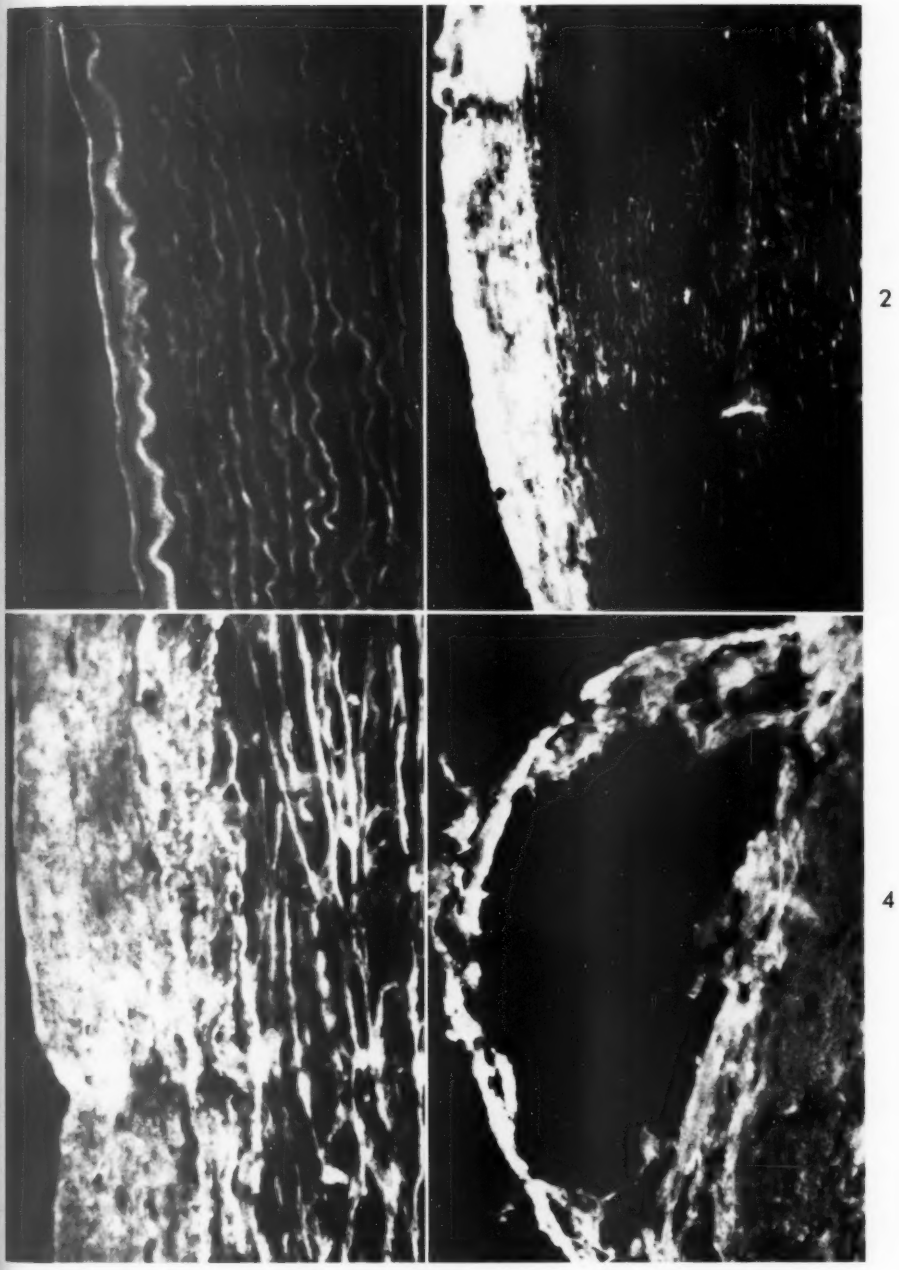


FIG. 4. Frozen section through an aortic fatty streak; adult of 43. In the process of washing, the endothelium has tended to lift from the underlying tissue and the lipophages which constituted the bulk of the intimal elevation have been washed out. Lying under the endothelium, however, are long coarse threads of brilliantly fluorescent material. × 175.

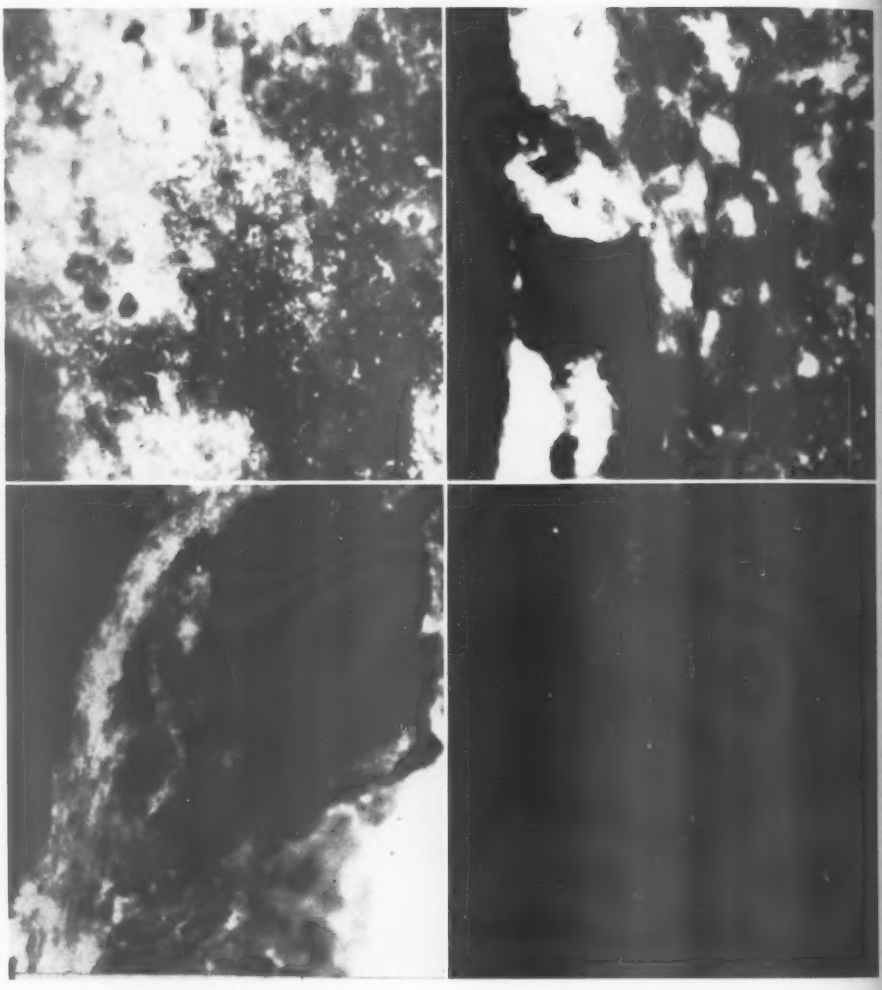


FIG. 5. Frozen section through a mature atheromatous plaque. There are large masses of fluorescent material lying within the substance of the plaque at different levels. The tissue lying in between these major aggregates shows relatively little fluorescence. × 650.

FIG. 6. Frozen section through a coarctation of aorta. The greatly thickened intima contains numerous coarse, irregular aggregates of specific fluorescent material. The left part of the field is occupied by a brilliantly fluorescent mural thrombus which can be seen to have been re-endothelialized. × 175.

FIG. 7. Frozen section of a fatty streak in a portion of the aorta with coarctation. The intima here contains abundant fluorescent material as usually seen in this type of lesion. × 175.

FIG. 8. A serial section from the same block shown in Figure 7. Pretreated with uncoupled anti-fibrin serum before exposure to fluorescein-coupled anti-fibrin serum. The brilliant fluorescence seen in Figure 7 has been almost entirely abolished here, indicating that the unconjugated serum has "blocked" the reacting sites in the section. This illustrates the immunologic specificity of the staining procedure. × 175.

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8

RHEUMATOID ARTHRITIS AND THE CELLULAR ORIGIN OF RHEUMATOID FACTORS

ROBERT C. MELLORS, M.D., PH.D.; ADAM NOWOSLAWSKI, M.D.,* AND LEONHARD KORNGOLD, PH.D.

From the Hospital for Special Surgery, Philip D. Wilson Research Foundation, affiliated with The New York Hospital-Cornell Medical Center, and the Department of Pathology, Cornell University Medical College, New York, N.Y.

The first pathologic change in rheumatoid arthritis[1] occurs in the synovial membrane, the lining of the joint capsule. In the active phase of the disease the synovial membrane is thrown into numerous folds and villous projections which cover, destroy, and replace the articular cartilage (Figs. 20 and 21). The hypertrophic villi with their richly vascularized fibrous cores are crowded with inflammatory cells, principally lymphocytes and plasma cells, and covered by stratified layers of proliferated synoviocytes. The lymphocytes have diffuse and nodular distributions.[2] The plasma cells include immature, mature and Russell-body types and have a diffuse distribution (Figs. 22 and 23).

When occurring elsewhere, as in lymph nodes and spleen, plasma cells are known to participate in immune responses and to produce antibodies[3–5] and gamma globulins.[6] In the synovial membranes and lymph nodes obtained from patients with active rheumatoid arthritis, plasma cells have been shown[7,8] to form and apparently to "secrete" rheumatoid factor, the unusual category of macroglobulins[9] found characteristically, if not exclusively, in the serum of individuals with this disease.[10] The serologic reactions for rheumatoid factor, such as the sheep-cell agglutination[11] and the precipitin[12] tests, utilize one or the other of two kinds of test proteins which form agglutinating or precipitating complexes with rheumatoid factor. The test protein in the sheep-cell test is rabbit immune complex, that is, sheep erythrocytes coated with rabbit antibody. The test protein in the precipitin test is human gamma globulin, in an aggregated[13–15] or altered state.

Specific reactants (stains) for the microscopic detection of rheumatoid factor in frozen sections of tissues—and for establishing the diagnosis of active rheumatoid arthritis in synovial biopsy specimens—can

This work was supported in part by a research grant from the National Institute for Arthritis and Metabolic Diseases, United States Public Health Service, and by the Bicknell Foundation.

Accepted for publication, May 8, 1961.

* Rockefeller Foundation Fellow. Present address: Department of Pathologic Anatomy, Medical Academy, Warsaw, Poland.

be prepared by conjugating either of these test proteins with fluorescent dyes.[7,8] Two fluorescent dyes are available for the labeling procedures, namely, apple-green fluorescein isothiocyanate[16] and orange-red lissamine rhodamine B 200.[17] Thus two stains for tissue rheumatoid factor can be prepared in contrasting colors. With these, not only the cellular origin but the number of rheumatoid factors, whether one, two, or possibly more, can be ascertained as reported herewith and as mentioned briefly in a previous publication.[8]

Material and Methods

Fluorescent Immune Complex

This was a soluble complex of rabbit antibody and homologous fluorescent antigen, fluorescein isothiocyanate-labeled bovine serum albumin, prepared as described elsewhere.[6]

Fluorescent Aggregated Human Gamma Globulin (Fluorescent Aggregate)

This was prepared by the heat aggregation[15] of lissamine rhodamine B 200-labeled human gamma globulin (Cohn fraction II, Pentex lot number 50 G 08). The conjugation procedure was that of Chadwick, McEntegart and Nairn,[17] slightly modified. To each ml. of protein solution (40 mg. of protein per ml. in isotonic saline) was added 1 ml. of 1 M, pH 9, carbonate buffer, followed by 0.1 ml. of lissamine rhodamine sulphonyl chloride solution, and, if needed to maintain alkalinity, 0.1 ml. additions of carbonate buffer. The conjugate was treated with washed, powdered activated charcoal (shown *not* to remove protein from solution) and dialyzed against isotonic saline buffered at pH 8 with 0.01 M phosphate.

Procedures

The preparation of frozen tissue sections, the fluorescence staining methods, the absorption of fluors with rabbit bone marrow powder, the proofs of immunospecificity, and the technique of fluorescence microscopy and photography were, with slight modification, the same procedures used previously.[8] The two reactants for rheumatoid factor, fluorescein-labeled immune complex and rhodamine-labeled aggregated human gamma globulin, were used as follows: simultaneously, after mixing in equal parts by volume; in sequence, with washing of sections in buffered saline before addition of the second fluor; and separately. For visual observation and full color photography of yellow-green (apple-green) and orange-red fluorescence, a GG 4 barrier filter was used in the fluorescence microscope, and for the selection of yellow-green a BG 23 filter was placed above the GG 4.

Source and Nature of Tissues

Tissues were obtained from 7 adult patients: 5 women and 2 men, ranging in age from 31 to 65 years. These had active rheumatoid arthritis of many years' duration and had received various forms of treatment, including corticosteroids (in one instance administered for 10 years). Diagnostic biopsy was made and surgical procedures performed in these patients by the professional staff of the hospital. The following specimens were provided for study: synovial tissues of wrist and hand, 5; and knee, 1; and axillary lymph node, 1. Tissue blocks, numbering 112, were cut from these specimens, frozen for special study, and fixed and embedded in paraffin for conventional study in the following number: synovial tissue, special, 53; conventional, 54; and lymph node, special, 1; conventional, 4. The pathologic diagnosis of synovial tissue was in each case chronic exudative and proliferative synovitis, histo-

logically compatible with rheumatoid arthritis. The lymph node showed follicular hyperplasia.

Control tissue from 9 patients, 12 to 69 years of age, 8 males and 1 female, without rheumatoid arthritis provided the following tissue blocks for study: synovial tissue, special, 6; conventional, 7; lymph nodes, special, 2; conventional, 5; spleen, special, 14; conventional, 2; and other tissues, special, 3; conventional, 2.

In the present study approximately 350 frozen sections were treated with fluorescent reactants, examined, and described, and more than 700 photomicrographs were made as a permanent record of the observations. This work comprised a part of a continuing investigation of cellular rheumatoid factor in which, to date, tissues from 24 patients with active rheumatoid arthritis and from 44 patients without this disease have been investigated. This material has included 19 specimens of active rheumatoid synovitis, 15 lymph nodes from patients with active rheumatoid arthritis, and, as controls, 25 synovial specimens and 56 other specimens, among them 13 lymph nodes, from patients without rheumatoid arthritis.

Results

Synovial Membranes in Active Rheumatoid Arthritis

The cells containing rheumatoid factor detectable with fluorescent reactants comprise a variable portion of the cell-rich inflammatory exudate which lies beneath the surface mesothelium of the thickened synovial membrane and in the stalks of the hypertrophic synovial villi. Whether stained with one or the other reactant—used separately, in mixture, or in sequence—the cells containing rheumatoid factor are plasma cells in various stages of development and include immature, mature, and Russell-body types (Figs. 1 to 13). Rheumatoid factor is formed almost exclusively in the cytoplasm of these cells (although occasionally a nuclear, or possibly nucleolar, focus of localization is also seen; Figure 7) and is apparently liberated into the cellular surroundings by the secretion-like shedding of cytoplasm.

When a tissue section is stained simultaneously with the two reactants (rhodamine-labeled aggregate and fluorescein-labeled immune complex), some of the synovial plasma cells are orange-red (Figs. 6, 7, and 9), as when reacting with fluorescent aggregate alone (Fig. 3); others are apple-green (Figs. 8, 12, and 17), as when stained with fluorescent immune complex alone (Figs. 1, 2, and 5); and some have a mixture of these colors (Figs. 15, 16, and 18). When the color mixture is homogeneous, the plasma-cell cytoplasm is yellow (Fig. 18), an additive effect formed by hues of orange and green; and if inhomogeneous, the plasma-cell cytoplasm is green or yellow in some regions and orange or red in others (Figs. 15 and 16). Thus a plasma cell may have either a "pure" color imparted by an individual fluorescent reactant or a mixture of colors derived from both reactants. Some microscopic fields contain plasma cells with identical colors, and other lesions are composed of plasma cells with a variety of colors. Although each synovial specimen

shows variation in cellular attributes from field to field and section to section, plasma cells, in general, stained orange-red with fluorescent aggregate are more numerous than those stained apple-green with fluorescent immune complex. Plasma cells stained with both reactants are the least frequent of all.

When a serial tissue section is treated first with fluorescent (orange-red) aggregate and then, after washing, with fluorescent (apple-green) immune complex, orange-red is the predominant color of stained plasma cells (Figs. 4 and 13), followed in frequency by yellow or some other color mixture (Fig. 19), and apple-green is not seen. If the sequence is reversed by staining a companion section first with fluorescent complex and then, after washing, with fluorescent aggregate, apple-green is the predominant color of plasma cells (Fig. 10), followed in frequency by orange-red (Fig. 11), and by yellow or some other color mixture. Lastly, prior treatment of a section with unlabeled aggregate (as also with rabbit antiserum against 19S human gamma globulin) blocks the subsequent staining reaction with fluorescent aggregate and with fluorescent immune complex; whereas prior treatment of a section with unlabeled immune complex blocks the subsequent staining reaction with fluorescent immune complex but not with fluorescent aggregate.

The logical interpretation of the results obtained with the simultaneous and the sequential staining procedures is that there are at least two cellular rheumatoid factors. One is detected only with fluorescent aggregate, and the other is demonstrable with fluorescent immune complex as well as with fluorescent aggregate. Some plasma cells form one rheumatoid factor, some form a second, and still others form both factors.

Lymph Nodes in Active Rheumatoid Arthritis

In hyperplastic lymph nodes obtained from individuals with active rheumatoid arthritis, two categories of cells, possibly belonging to the same family, contain rheumatoid factor detectable with fluorescent reactants: (a) germinal-center cells and (b) internodular plasma cells of immature, mature, and Russell-body types. As in synovial membrane, cells stained orange-red with fluorescent aggregate are generally more numerous than those stained apple-green with fluorescent immune complex. When stained, germinal-center cells are either orange-red (Fig. 14) or apple-green throughout, and color mixtures are not seen within a particular center. Internodular plasma cells are orange-red, apple-green, or mixtures of these colors. The results and the interpretations obtained by staining with the reactants in sequence and by the use of blocking procedures are the same as described in the synovial membrane.

Control Tissues

To date more than 1,700 immunofluorescence reactions have been carried out on frozen sections of tissues obtained from 24 patients with active rheumatoid arthritis and from 44 patients without this disease. In each of the 19 specimens of active rheumatoid synovitis examined, cellular rheumatoid factor was demonstrated, as also in each of 15 lymph nodes obtained from patients with active rheumatoid arthritis. Cellular rheumatoid factor was not detectable in any of the 22 synovial specimens obtained from patients with other forms of synovitis and arthritis (infectious, tuberculous, degenerative, traumatic, gouty, villonodular, and nonspecific) nor, with one exception, in any of the other 56 control specimens, including 13 lymph nodes, that have been studied. The exception was an unusual example of Waldenström's macroglobulinemia,[18] in which immature plasma cells in lymph node and spleen were stained with fluorescent aggregate but not with fluorescent immune complex.[8]

Discussion

In an analogy with the two categories of reactants used in the serologic tests for rheumatoid factor, two fluorescent reactants have been prepared and used for the detection of rheumatoid factors *in situ* in tissue sections: rhodamine-labeled aggregated human gamma globulin and fluorescein-labeled rabbit immune complex. With these reactants it has been shown that plasma cells in the synovial membrane, and, in lymph nodes, germinal-center and internodular plasma cells are the sites of origin of rheumatoid factors in individuals with active rheumatoid arthritis. It has been established that there are at least two cellular rheumatoid factors: one, the more abundant, is detected only with fluorescent aggregate; the other is demonstrable with fluorescent immune complex as well as with fluorescent aggregate. Plasma cells form either one or the other factor, or both simultaneously. Germinal-center cells synthesize either one or the other factor but apparently not both in the same center. The multiplicity of cellular rheumatoid factors is in keeping with the heterogeneity of serum rheumatoid factors as shown by precipitin reactions[19,20] and ion-exchange chromatography.[21,22]

While the precise nature and function of rheumatoid factor are unknown, 3 alternatives have been considered: an increased concentration of a normal serum globulin; an abnormal globulin; and an antibody against an as yet undetermined antigen. Several bits of evidence have been cited in favor of the latter possibility. Rheumatoid factor is a gamma$_1$ macroglobulin,[23] a class of proteins known to have antibody function. Rheumatoid factor reacts only with the gamma globulins of man and a few other species, and the gamma globulin must be either

aggregated or adsorbed onto larger particles or be in the form of an immune (antigen-antibody) complex. The gamma globulins of other species, such as the rabbit immune complex, behave in some respects [19] similarly to the cross-reacting antigens of the classical immunologic systems. Rheumatoid factor is formed by the family of cells which produces antibodies,[4,5] 7S gamma globulin,[6] and 19S gamma globulin.[7,8,24] Thus the cellular origin, as well as the immunochemical properties, of rheumatoid factor suggest an antibody-like nature and function.

In that event, several possibilities as to the hypothetic antigen should be considered. The antigen may be a product or component of a bacterial or viral pathogen which has antigenic determinants in common with aggregated or altered (but not native) gamma globulins. The antigen may be a foreign substance in combination with human tissue or serum component, the latter undergoing sufficient alteration to become antigenic in the host. The antigen may be a tissue or serum component, such as gamma globulin, altered by an unknown mechanism to make it autoantigenic. The antigen may be an unaltered tissue or serum component which has become auto-antigenic in the presence of adjuvant-like substances, or as a consequence of weakness of immunologic homeostasis or because of proliferation of forbidden clones of antibody-forming cells.[25] (Arising as from a center of growth, enlarging as a nodule, and forming one or the other type of rheumatoid factor, a germinal center in lymph node or spleen appears morphologically and functionally to comprise a clone of cells—a family of cells arising from one parent cell).

Thus the studies of serum and cellular rheumatoid factors focus attention on, and stimulate a search for, extrinsic or intrinsic antigens which may play important roles in the pathogenesis of rheumatoid arthritis.

Summary and Conclusions

Comparable to the two categories of reactants used in the serologic tests for rheumatoid factor, specific reactants (stains) were prepared for the microscopic detection of rheumatoid factor *in situ* in tissue sections: rhodamine-labeled aggregated human gamma globulin and fluorescein-labeled rabbit immune complex.

Frozen sections of synovial membranes and lymph nodes obtained from patients, principally adults, with active rheumatoid arthritis were treated with the fluorescent reactants in simultaneous, sequential, and separate staining procedures. To date, tissue rheumatoid factor has been demonstrated in each of the 19 specimens of active rheumatoid synovitis examined, as also in each of 15 lymph nodes obtained from patients with active rheumatoid arthritis.

Plasma cells in the synovial membranes, and germinal-center cells and internodular plasma cells in lymph nodes, were the sites of origin of rheumatoid factor. The results obtained with the simultaneous and sequential staining procedures (performed on tissues from 7 adults with active rheumatoid arthritis) indicated that there were at least two cellular rheumatoid factors: one, generally the more abundant, was detected only with fluorescent aggregate; the other was demonstrable with fluorescent immune complex as well as with fluorescent aggregate.

Some plasma cells formed one rheumatoid factor, some formed a second, and still others produced both factors. Germinal-center cells synthesized either one or the other factor but not apparently both in the same center.

The plasma cell origin, as well as known immunochemical properties, of rheumatoid factor suggests an antibody-like nature and function and stimulates a search for extrinsic or intrinsic antigens which may play important roles in the pathogenesis of rheumatoid arthritis.

Cellular rheumatoid factor was not detectable in any of the 22 synovial specimens obtained from patients with other forms of synovitis and arthritis (infectious, tuberculous, degenerative, traumatic, gouty, villonodular, and nonspecific) or, with one exception reported previously, in any of the 56 control specimens, including 13 lymph nodes, that were studied.

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We acknowledge our appreciation to Dr. Lee Ramsay Straub, Dr. Richard H. Freyberg, Dr. Philip D. Wilson, Jr., Dr. Ralph Heimer, Dr. Josué Corcos, and other members of the professional staff of the hospital for their cooperation during the course of this study, and extend our thanks to Dr. Beth Sengson and Mr. Alfred Stoholski for their valuable assistance.

[*Illustrations follow*]

Legends for Figures

Figures 1 to 19 are fluorescence photomicrographs of cells in frozen sections stained with fluorescent reactants (rhodamine-labeled aggregate and fluorescein-labeled immune complex) for rheumatoid factors. Unless otherwise specified, all magnifications are 1,500 times. The tissues, synovial membranes, with one exception (Fig. 14), were obtained from patients with active rheumatoid arthritis.

Figs. 1 and 2. A mature plasma cell with homogeneous, apple-green cytoplasm contains rheumatoid factor and an eccentric nucleus devoid of color. Fluorescent immune complex (single stain).

Fig. 3. A mature plasma cell with an indistinctly homogeneous orange cytoplasm contains rheumatoid factor. Fluorescent aggregate (single stain).

Fig. 4. A mature plasma cell contains cytoplasmic rheumatoid factor (orange color). Fluorescent aggregate followed by fluorescent complex (sequential stains).

Fig. 5. Russell-body plasma cells contain rheumatoid factor in the cytoplasmic spherical bodies (green color). Fluorescent complex.

Fig. 6. An immature plasma cell contains rheumatoid factor in its cytoplasm (orange color). Fluorescent aggregate and fluorescent complex (paired stains).

Fig. 7. A mature plasma cell contains rheumatoid factor in its cytoplasm and nucleus (orange color). Fluorescent aggregate and fluorescent complex (paired stains).

Fig. 8. A mature plasma cell contains cytoplasmic rheumatoid factor (green color). Fluorescent aggregate and fluorescent complex (paired stains).

Fig. 9. Two plasma cells with tiny Russell bodies near the limit of optical resolution contain rheumatoid factor (orange color). Fluorescent aggregate and fluorescent complex (paired stains).

Fig. 10. A mature plasma cell contains rheumatoid factor in its cytoplasm (green color). Fluorescent complex followed by fluorescent aggregate.

Fig. 11. An immature plasma cell contains cytoplasmic rheumatoid factor (orange color). Fluorescent complex followed by fluorescent aggregate.

Fig. 12. A Russell-body plasma cell contains cytoplasmic rheumatoid factor (green color). Fluorescent aggregate and fluorescent complex (paired stains).

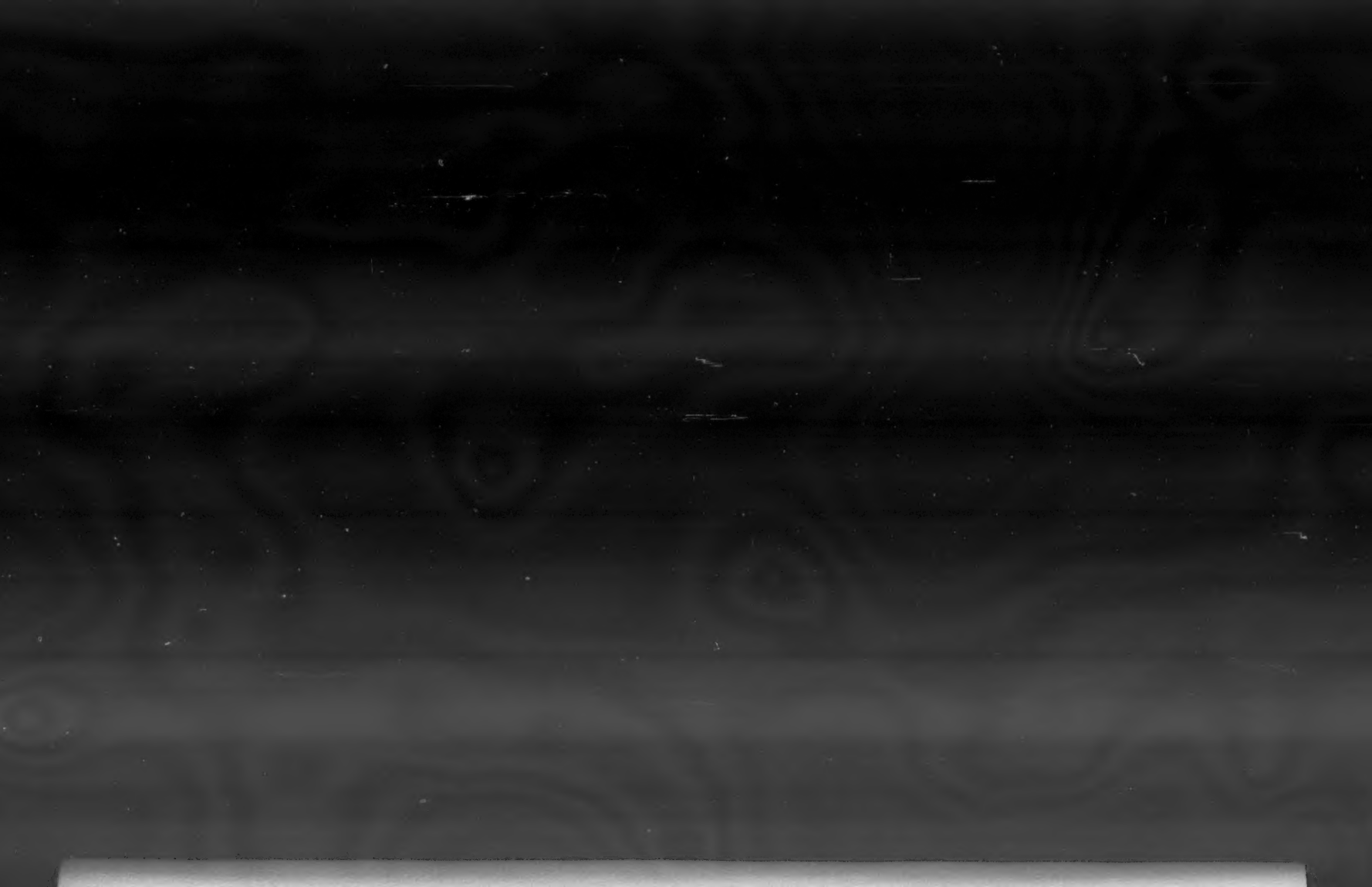
Fig. 13. Plasma cells contain cytoplasmic rheumatoid factor (orange to orange-red color). Fluorescent aggregate followed by fluorescent complex. × 520.

Fig. 14. Lymph node. Germinal-center cells contain rheumatoid factor (orange). Fluorescent complex followed by fluorescent aggregate. × 325.

Figs. 15 and 16. A mature plasma cell contains both rheumatoid factors in its cytoplasm (green, yellow, and red). Fluorescent aggregate and fluorescent complex (paired stains). Fig. 16: × 1,275.

Fig. 17. A bizarre and secretion-like shape of a plasma cell with cytoplasmic rheumatoid factor (green). Fluorescent aggregate and fluorescent complex (paired stains).

Fig. 18. Mature plasma cells (6 in a row) contain both rheumatoid factors in their cytoplasm (yellow color). Fluorescent aggregate and fluorescent complex (paired stains). × 612.



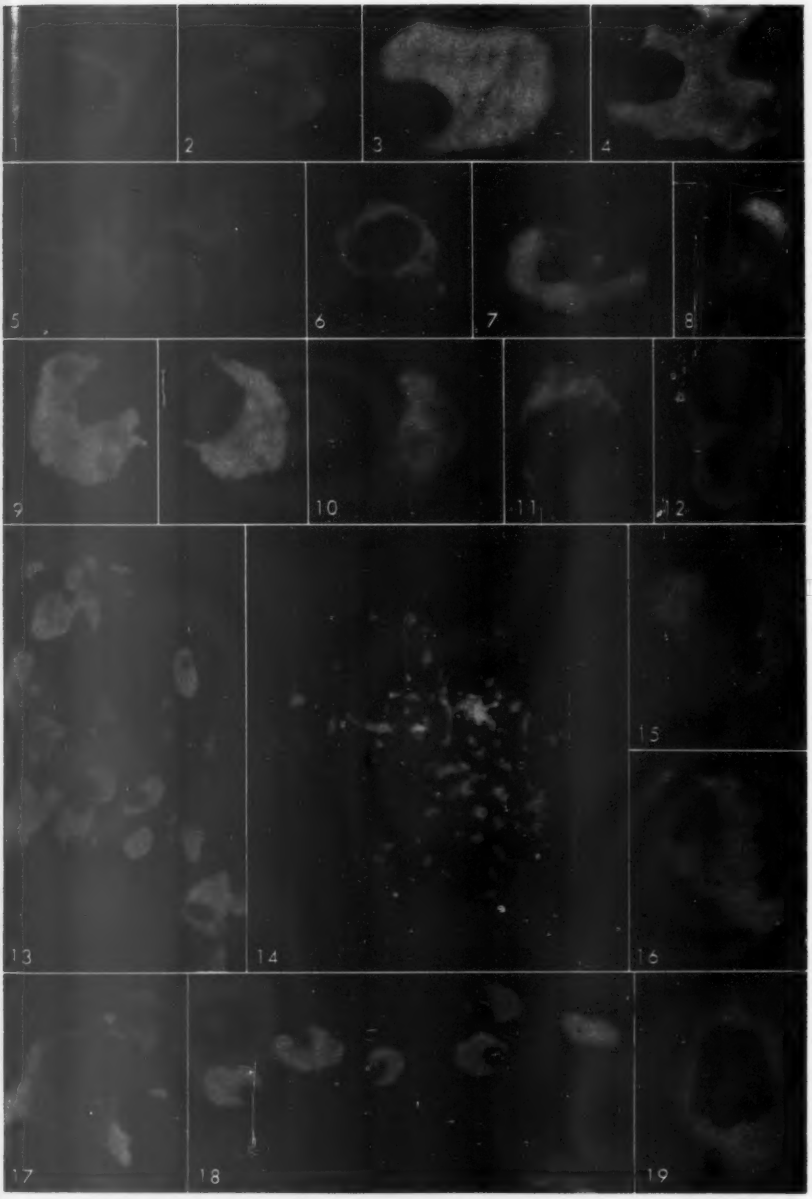
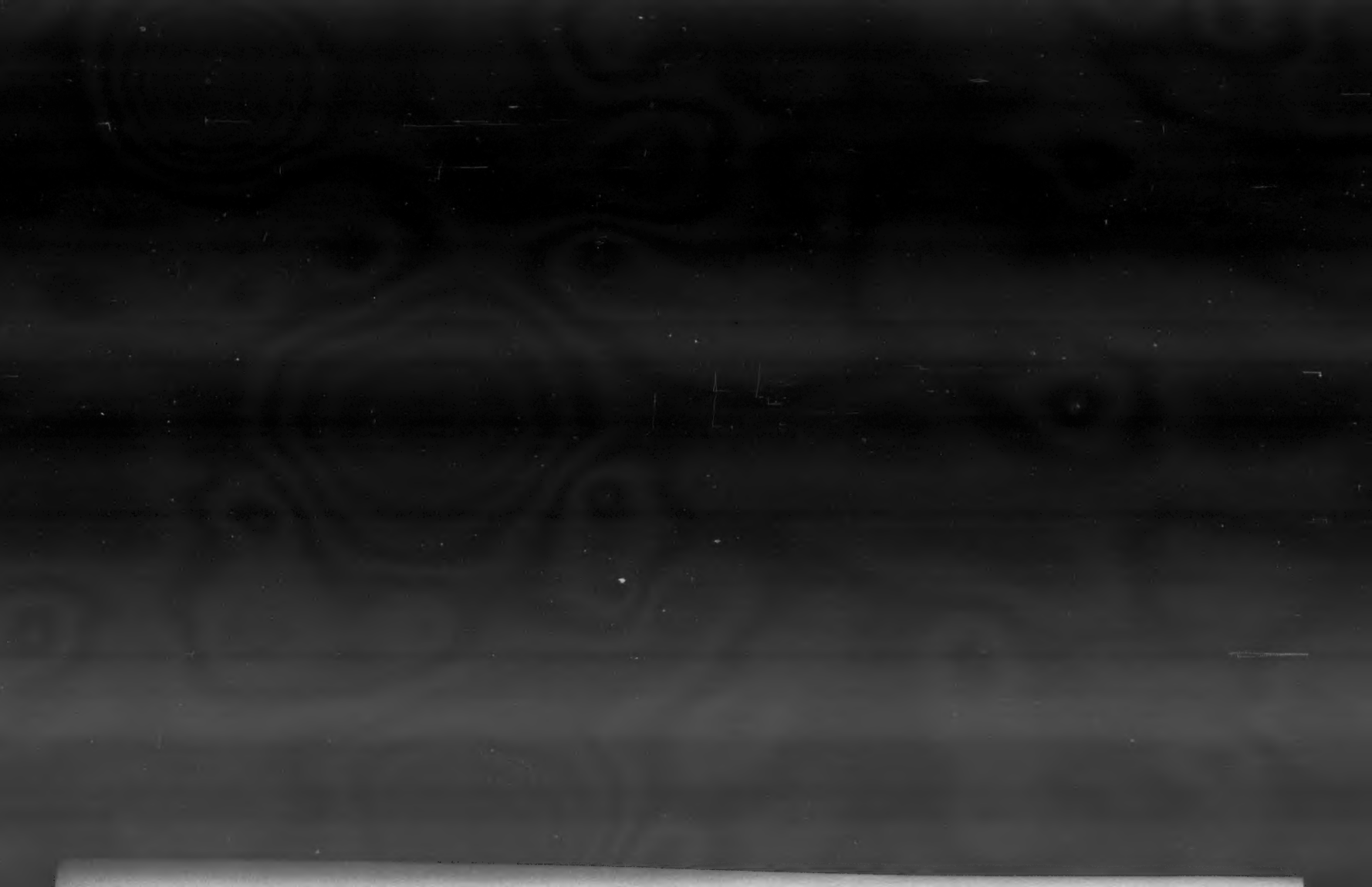


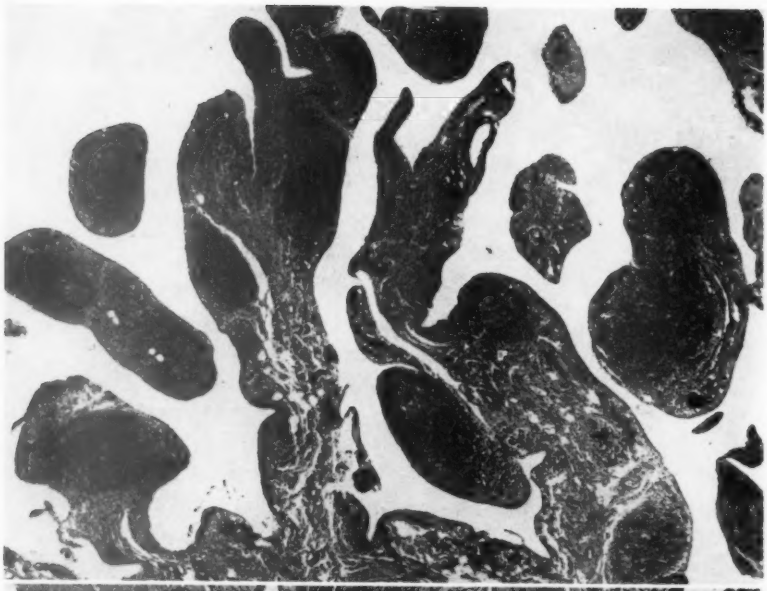
FIG. 19. A mature plasma cell contains both rheumatoid factors in its cytoplasm (green, yellow, and red). Fluorescent aggregate followed by fluorescent complex.

Figures 20 to 23 were prepared from paraffin sections stained with hematoxylin and eosin.

FIG. 20. The synovial membrane in active rheumatoid arthritis shows hypertrophic villi and chronic inflammatory cells with nodular and diffuse distributions. × 31.

FIG. 21. The articular cartilage of a joint in rheumatoid arthritis shows replacement by fibrous inflammatory tissue of overriding synovial membrane. × 48.





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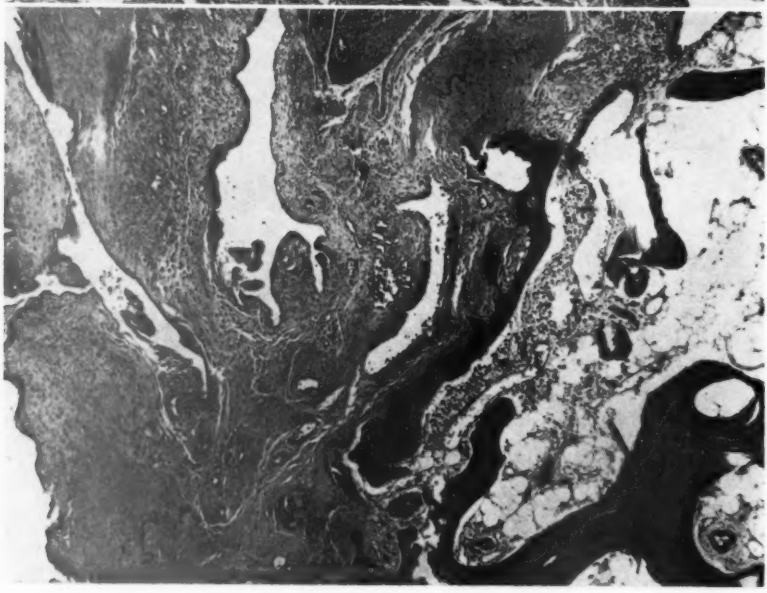

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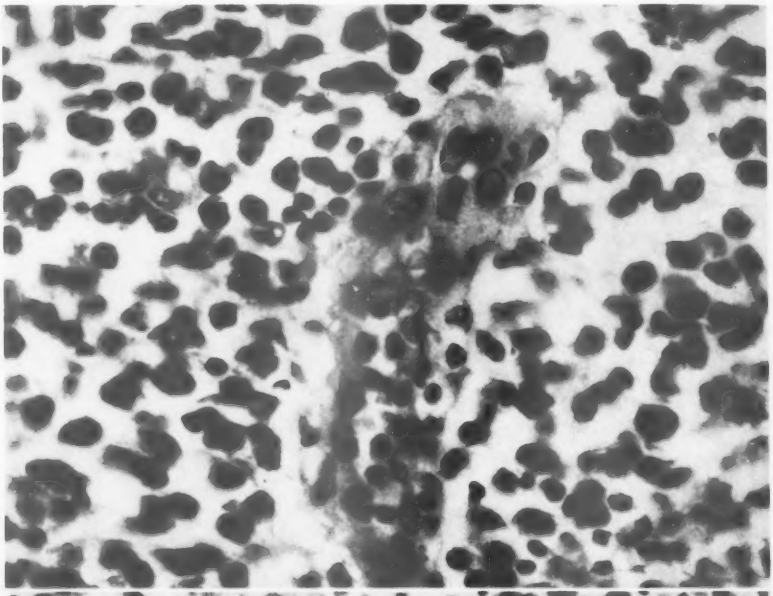

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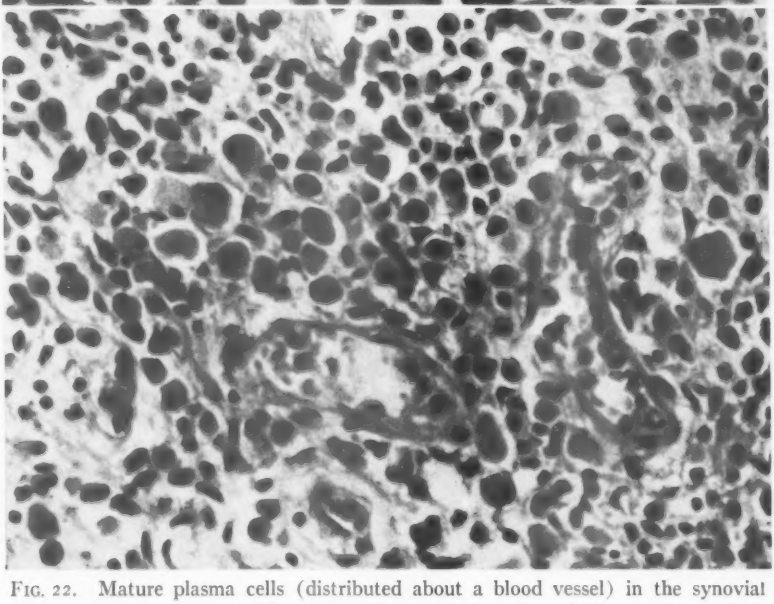
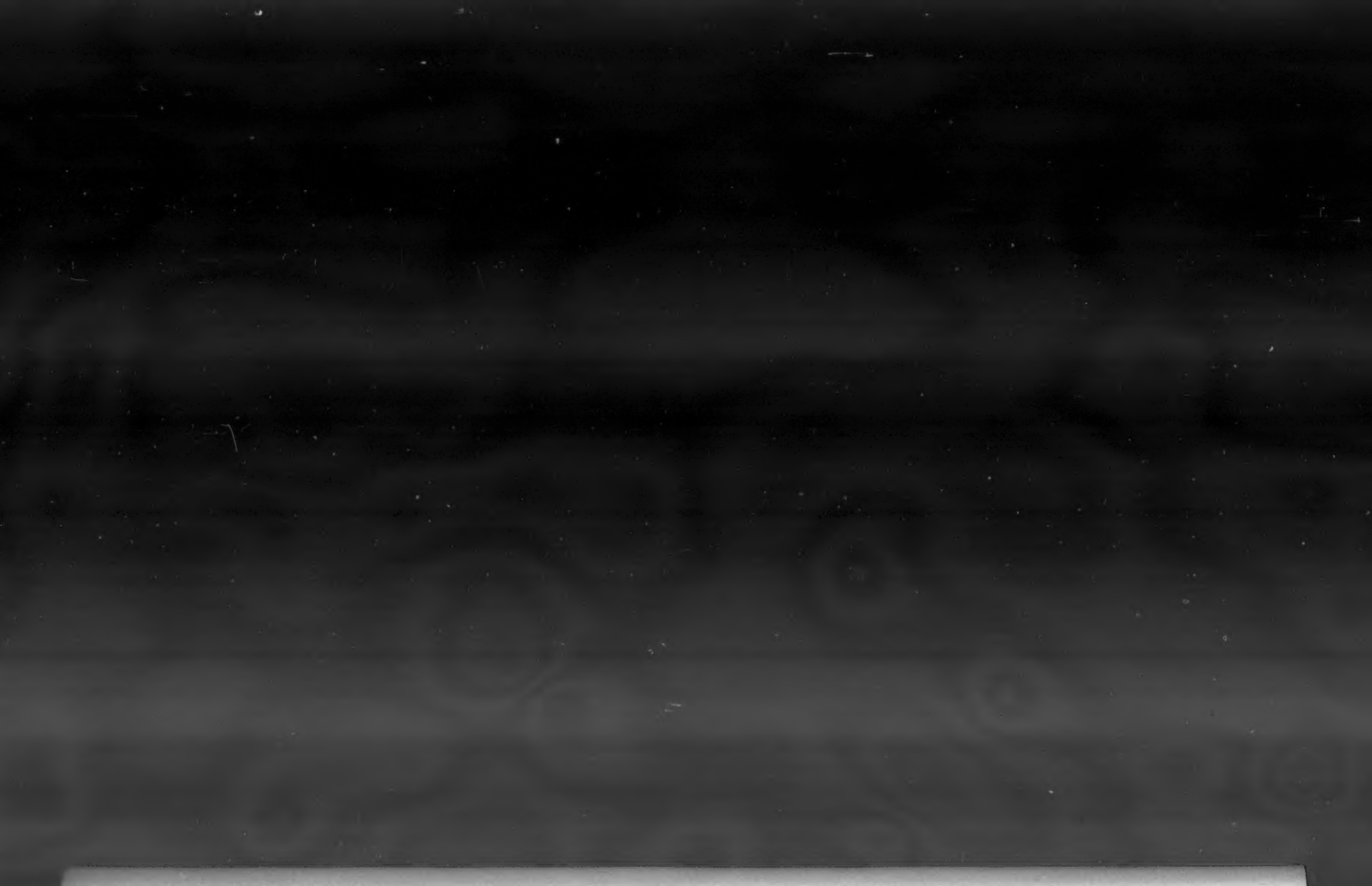


FIG. 22. Mature plasma cells (distributed about a blood vessel) in the synovial membrane in rheumatoid arthritis. These cells have basophilic cytoplasm (dark in black and white illustration, blue in full color). × 780.

FIG. 23. Russell-body plasma cells and other mononuclear cells are present in the synovial membrane in rheumatoid arthritis. The Russell bodies are principally of confluent type, one near an edge of the field is prismatic (roughly hexagonal), and all are acidophilic (gray in black and white illustration, red in full color). × 475.



INFRA-GLOMERULAR EPITHELIAL REFLUX IN THE EVOLUTION OF SEROTONIN NEPHROPATHY IN RATS

DOUGLAS WAUGH, M.D., AND HANNAH BESCHEL, DR. MED. UNIV.

From the Departments of Pathology, Queen's University and Hotel Dieu Hospital, Kingston, Ontario, Canada

The principal objective of this study was to plot the sequence of morphologic events in the evolution of acute renal failure induced experimentally in rats by the intraperitoneal injection of 5-hydroxytryptamine (serotonin). In an earlier study[1] it was found that serotonin injected intraperitoneally in rats produced variable degrees of necrosis of distal and proximal convoluted tubules in a very wide range of severity that appeared only partly related to the dosage of serotonin used. In the preliminary investigation, the experimental interval was kept relatively constant between 2 and 4½ days, while a wide range of dosages was employed. In the experiments reported below, the dose and route of adminstration of serotonin were unvaried while the experimental interval ranged from 3 hours to 3 weeks. In this way the sequence of necrobiotic changes in renal tubule epithelium has been plotted, as have the phenomena of recovery from ischemic damage.

In the course of the investigation, a lesion of the renal corpuscle was encountered that was first considered to be unique. Dr. M. Wachstein has since drawn attention to earlier descriptions[2,2] of the infra-glomerular cellular reflux phenomenon, a lesion whose pathogenesis and significance remain to some extent obscure.

MATERIAL AND METHODS

A total of 275 apparently healthy female adult white Wistar rats (purchased from the Canadian Breeding Laboratories, St. Constant, Quebec), weighing between 150 and 300 gm. at the start of the experiments, was used. Of these, 122 received intraperitoneal injections of 5-hydroxytryptamine creatinine sulfate,* and 106 control animals were injected with equivalent volumes of sterile physiologic saline. Experimental and control groups generally consisted of 6 animals each. The findings in the remaining 47 animals had to be disregarded because of deterioration of serotonin (35 animals) or spoilage of tissue by a breakdown in technical processing (12 animals). The rats were weighed on delivery to the laboratory, at the time of injection, and before sacrifice. They were housed, fed and killed in the manner described earlier.[1] Serotonin and physiologic saline were prepared and administered in the manner previously described.[1] Two per cent serotonin was used in the majority of

Supported by a Grant-in-Aid from the National Research Council of Canada, Ottawa, Ontario.

Accepted for publication, June 8, 1961.

* Supplied in powder form and also purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio.

groups, but a 1 per cent solution was employed in 33 animals in order to determine whether the concentration of the injected serotonin had any bearing on the incidence or severity of renal lesions. A dosage of 30 mg. per kg. of body weight was used in 15 groups of animals and 13 mg. per kg. in 5 groups. Three animals received injections of 1 per cent serotonin in dosages of 202.5 mg. per kg., 232.5 mg. per kg., and 216 mg. per kg., respectively.

No anesthesia was used at the time of injection in 13 groups (78 animals) or for their controls (82 animals). To evaluate the possible adjuvant effect of anesthesia, 8 groups totaling 38 animals were subjected to light ether narcosis at the time of injection. Twenty-seven animals served the purpose of determining an optimum safe dose for the production of renal lesions. The possible influence of serotonin concentration in the injected fluid on the production of lesions was studied in 11 animals and their 16 controls.

Groups of experimental and control animals were killed at the following intervals after injection: 3, 6, 12 hours, 1, 2, 4, 6, 8, 10, 14 and 21 days. One animal was killed 2½ hours following injection and is included in the "3-hour" group.

All necropsies were performed immediately after death. At necropsy, the abdomen and thorax were opened, the viscera were inspected, and the kidneys, together with adrenals, were removed at once. Individual kidney weights were recorded, and the gross appearance of the kidneys was noted. From 116 experimental and control animals, sections of one kidney were fixed in 2 per cent buffered osmic acid and preserved for examination by electron microscopy. This portion of the study is not yet complete and will be reported later.

All other kidneys were cut longitudinally, and after the gross appearance of the cut surface was noted, they were fixed in 10 per cent neutral buffered formalin for 48 hours with a change of formalin after 24 hours. The weights of the eviscerated carcasses were recorded, and any gross alterations of the organs were noted.

Microscopic sections cut at 5 μ were stained with hemalum-phloxine-saffron (HPS). The periodic acid-Schiff (PAS) or trichrome methods were used in selected cases. Oil red O was used for staining frozen sections to demonstrate fat.

Results

None of the 106 control animals developed renal lesions of the type found in those treated with serotonin. The following abnormalities were found in the kidneys of 9 control animals: unilateral hydronephrosis in 1, interstitial lymphocytic infiltration in 6, and small areas of cortical scarring in 2.

The great majority of animals injected with serotonin manifested signs of illness or discomfort within 5 to 10 minutes following injection. Cyanosis, especially noticeable on the feet, dyspnea and flaccidity were the principal manifestations of discomfort. Cyanosis and dyspnea usually lasted 1 hour and lassitude for 2 hours. Response to irritant stimuli remained reduced for 3 to 4 hours in several cases. The animals that remained clinically well after serotonin injection were invariably those who subsequently proved to have little or no renal damage.

At necropsy, the ratio of kidney weight to eviscerated carcass weight was calculated as in the earlier experiments (K/C Index).[1] For the 122 serotonin-treated animals, this index averaged 6.2, with a range of 5.31 to 7.35, while the mean value for 106 controls was 5.62 (range 4.94 to

6.34). The renal enlargement in the treated animals was associated with cortical pallor and mottling with irregular reddish areas, or confluent and generalized pallor where renal damage was severe. Those animals killed 3 hours after serotonin injection had pale, slightly bulging renal cortices, whereas those killed 2 or 3 weeks after injection demonstrated irregular, wedge-shaped, depressed cortical scars. The cortical pallor, when present, usually affected the entire depth of the cortex, and in the most severe lesions there was also involvement of the outer medulla. Sectors of grossly normal renal parenchyma, when these were apparent, usually corresponded with the axes of major vessels within the kidney. Similar sparing was also common in the parahilar cortex and at the poles of the kidneys.

Microscopic examination was carried out with the same methods and grading criteria described earlier.[1] The serotonin-treated animals in the present experiments showed both focal and widespread necrosis of renal tubules, with subtotal cortical necrosis occurring in a few animals. As in other studies, isolated animals in various groups failed to develop any perceptible renal lesion. The over-all incidence of renal damage was 67.2 per cent (82 of 122 treated animals). Only one animal died following serotonin injection.

In the group killed 3 hours after 30 mg. per kg. of 2 per cent intraperitoneal serotonin, the principal manifestations of damage were "early" necrobiotic phenomena. The most interesting lesion, found in 5 of the 6 animals, consisted of a reflux of dying epithelium from the infra-glomerular segment of the proximal convoluted tubule into Bowman's space (Figs. 1 and 2). At this stage the refluxed epithelial cells were shrunken and their nuclei pyknotic. There was no visible attachment of the cells, except to one another. The displaced epithelium usually formed a bulky mass which appeared to displace the otherwise intact glomerulus. The lesion of epithelial reflux was found in all of the 4 animals with renal damage in this group. Bowman's spaces in these same 4 animals also contained a flocculent, acidophilic substance (Fig. 4). In 2 of them some of the Bowman's spaces contained homogeneous, pale protein precipitate, usually perforated by large, clear, rounded vacuoles (Fig. 5). Below the point of epithelial detachment in the upper proximal tubules, there was pronounced pallor of the basal cytoplasm in the epithelium, with irregular enlargement of the cells (Fig. 2). At this stage the pale areas in the cytoplasm were not sharply defined, and they were interpreted as cellular edema rather than as vacuoles, except in one animal where definite vacuoles were noted. Stains for glycogen and fat failed to demonstrate either substance in the affected cells. In addition there was extensive but variable

pyknosis of epithelial nuclei. Associated with this, rounded, dense, basophilic droplets were numerous in the lumens of the tubules, and were interpreted as either extruded pyknotic nuclei or nuclear fragments (Fig. 6).

All 6 animals killed 6 hours after serotonin had renal lesions. The 4 with the most severe damage all demonstrated the epithelial reflux lesion seen in the "3-hour" group. The phenomena of cellular edema, nuclear pyknosis and intraluminal basophilic droplets were also common. The principal difference between the kidneys of animals of this group and those killed at 3 hours was the evidence of more advanced cellular breakdown and accumulation of both nuclear and cytoplasmic debris in the tubular lumens. Cellular debris tended to be concentrated in the tubules of the outer medulla rather than being diffusely scattered through cortical and medullary tubules as in the "3-hour" group. A localized area of hemorrhagic necrosis of the bowel was present in one animal, possibly the result of trauma at the time of injection.

Of the 6 animals in the group killed 12 hours after serotonin, one showed no nephropathic effect. In the remaining animals, fully developed necrosis of tubule epithelium (Fig. 3), with loss of nuclear staining, was the outstanding manifestation of damage. The changes of nuclear pyknosis, basophilic droplets, and intraluminal debris were noted as in the preceding groups. In 2 animals, epithelial cells of the tubules manifested multiple, large, clear, rounded, fat-filled vacuoles in their basal cytoplasm. The cells so affected appeared otherwise unaltered. Both hyaline and granular casts occupied the lumens of many medullary tubules. The kidney in one animal of this group exhibited what was interpreted as subsiding reflux lesions (Fig. 4). There was only slight protrusion of tubule epithelium into Bowman's space, but the cells now appeared completely necrotic and nuclear staining was absent. The reflux lesion could not be identified in animals killed later than 12 hours after serotonin, although epithelial necrosis or regeneration were noted here, depending upon the post-serotonin interval.

In the 6 animals killed one day following serotonin, pyknosis of epithelial nuclei had largely disappeared, and the necrotic cells were homogeneously acidophilic (Fig. 7). This damage could now be seen to be localized mainly to the proximal tubules though, with more severe lesions, both proximal and distal tubules were affected. Basophilic droplets and lumen debris were now heavily concentrated in tubules just inside the corticomedullary junction, as were casts similar to those in the earlier groups. It could also be noted that surviving tubules tended to be concentrated close to large empty venous channels (Fig. 7). Basal epithelial vacuoles were common in otherwise intact tubule lining cells as in the 12-hour group.

All 6 of the animals killed 2 days after serotonin had renal damage. In one, the only alterations were occasional basal fat vacuoles in tubule epithelium and a few casts. In the remainder, basophilic droplets, necrosis, vacuoles and casts were common. Basophilic droplets and intraluminal debris in the corticomedullary junction were decidedly less prominent than in earlier groups, and the number of casts was quite variable. A new feature at this stage was the appearance of abundant mitotic regeneration of tubule epithelium (Fig. 8), localized mainly in the deeper zones of the cortex. Mitotic figures were usually regular and normal in appearance, though polyploid figures were not infrequent (Fig. 9). Speed of regeneration was reflected in a mitotic frequency of up to 10 in a single high power field. The cytoplasm of the regenerating epithelium was usually pale, slightly basophilic and bulky, leading to a reduction in lumen caliber. A few tubules seemed to be slightly dilated at this stage. Minor interstitial infiltrations with lymphocytes and monocytes were also seen, usually in proximity to dilated tubules.

In the 5 animals killed 4 days after injection, dilatation of tubules in the areas of damage was the outstanding feature (Fig. 10). The epithelium lining the dilated tubules was lower than normal, and brush borders were not appearent. Nuclei were slightly enlarged, often causing local bulges into the lumen. The cytoplasm of these cells remained slightly basophilic and was more deeply stained than in the preceding group. Mitotic figures remained in evidence but were less frequent than in the animals killed 2 days after serotonin. Loose acidophilic debris could be found in the lumens of dilated tubules, but clearing of debris was obviously advanced in comparison with earlier groups. Focal exuberant epithelial proliferation was relatively common, with the formation of local protrusions or epithelial polyps in the lumens of tubules (Fig. 11). Rounded cell clusters apparently lying free in the lumens of tubules may have been the apexes of polyps or detached cell clusters on their way to elimination or impaction elsewhere. Foci of necrosis of tubules could still be identified at this stage, but were decidedly less frequent than in earlier groups. Intraluminal basophilic droplets were found in only 1 of the 5 animals. Focal cortical and corticomedullary interstitial infiltrations with lymphocytes and monocytes were noted in all gradable kidneys and minor interstitial edema was present in 3. Beginning peritubular and pericapsular fibrosis about Bowman's capsule were also noted.

The groups of 6 animals each, killed 6, 8, 10, 14 and 21 days respectively after serotonin, showed progression of the phenomena of healing described in the 4-day group. Peritubular and pericapsular scarring became more pronounced, and dilatation of tubules became less evident but had not entirely disappeared at 3 weeks. Residual necrosis was

present in 2 animals killed 6 days after serotonin and in 1 after 10 days. Eosinophilic debris in the lumens of tubules persisted up to 2 weeks, though in progressively diminishing amounts, and had disappeared entirely at the end of 3 weeks. Casts remained for a similar period though there was a progressive decrease in their number. Basophilic droplets were still present in one animal at 6 days and in 2 at 10 days. The droplets in these animals were more variable in size and more deeply stained than in animals killed earlier.

Dilatation of tubules was found in all groups of animals killed between 4 and 21 days, though the appearance of the dilated tubules changed as the lesions aged. The diameter of the dilated tubules became slowly reduced, and the lining epithelium, so strikingly flat at 4 days, gradually increased in height. The cytoplasm of the regenerated cells remained basophilic, and their slightly enlarged nuclei became closely crowded (Fig. 11). The beginning peritubular fibrosis at 4 days became increasingly evident, and the impression was gained that fibrosis may have played a constricting role in the reduction of dilatation. Similar fibrosis of Bowman's capsule was increasingly prominent with advancing time. Some dilated tubules were still apparent, however, even after 3 weeks, and these continued to have a lining of flattened epithelium. A few mitotic figures could still be found at 6 to 10 days after serotonin, but cell division was inconspicuous thereafter. The focal epithelial masses and polyps, so prominent at 4 days, were found in 2 animals killed 6 days after treatment, but were not seen in any of the animals sacrificed subsequently.

Focal interstitial infiltrations of the cortex and outer medulla, with lymphocytes and monocytes, were found in all groups killed from 4 to 21 days after serotonin and became quite prominent. Such infiltration tended to be concentrated around newly regenerated tubules, though this was difficult to assess with accuracy. Interstitial edema accompanied this cellular infiltration in 2 animals on the sixth to eighth days. Zones of interstitial infiltration and fibrosis tended to coincide with wedge-shaped, dimpled scars on the cortical surface.

To determine whether the light ether anesthesia that was used at the time of serotonin injection in earlier experiments[1] might have influenced the production of lesions, a group of 11 animals was subjected to light ether narcosis prior to injection, and the results compared with those in 6 otherwise similar animals not subjected to anesthesia. The renal lesions in the 2 groups were comparable, both in severity and stage of evolution, when the animals were killed 36 hours after 30 mg. per kg. of 2 per cent serotonin.

The possible influence of the concentration of injected serotonin was

tested in 6 groups of 6 animals each and their controls. These were killed at intervals of 1, 2, 4 and 6 days after serotonin at dosages of both 13 and 30 mg. per kg. of body weight and in concentrations of both 1 per cent and 2 per cent. When the same total dose per kg. of body weight was injected, there was no discernible difference in the incidence of lesions or their stage of evolution. In the course of determining an optimum safe dose for the production of renal lesions, 3 animals were injected with high doses of serotonin (202.5 mg. per kg., 232.5 mg. per kg., and 216 mg. per kg.). Lesions found in these animals were of the severe type and demonstrated pathologic changes analogous to those seen in other animals killed after the same time interval.

Discussion

The results of this study are in general agreement with the observations of others[1–7] in once more indicating the nephropathic effects of the vasospastic ischemia[6] induced by serotonin. In an earlier study[1] it was found that the principal renal effect of serotonin was the production of a variable amount of necrosis in both distal and proximal convoluted tubules, ranging in severity from the minor focal necrosis resembling that of human acute nephrosis[8] to extreme diffuse damage equivalent to symmetrical cortical necrosis in man. It was also noted that the early alteration of epithelial necrosis in renal tubules was followed rapidly by mitotic regeneration, and that as the tubules became relined by epithelium, they underwent striking dilatation.[1,2,9–11] The primary purpose of the present series of experiments was to determine the sequence of morphologic events prior to and following the changes of regeneration and dilatation of tubules.

The first point of interest in the evolution of the renal damage resulting from serotonin was the curious and transient lesion of epithelial reflux into Bowman's space. When this was first encountered, the possibility was entertained that it might have been an artifactual result of undue roughness during postmortem handling of the kidney. It was soon apparent that an artifact would be unlikely to be so consistently found only in the 12 hours following serotonin, never to appear again. It was also noted that the epithelium that regurgitated into Bowman's space was relatively well preserved 3 hours after serotonin, although the cells had the pyknotic nuclei and dense acidophilic cytoplasm indicative of early necrobiosis. This was more pronounced after 6 hours; at 12 hours, although epithelial displacement had largely disappeared, the cells were now completely necrotic and nuclear staining was lost entirely (Fig. 3). The evolution of this lesion was both precise and short-lived.

From these findings, it seems probable that the epithelial reflux lesion

is produced by the rapid and nearly simultaneous development of necrosis and detachment of the affected cells and their upward displacement by edematous enlargement of cells lower down the proximal tubule (Fig. 2). Whether or not the upward displacement of epithelium is opposed by any downward flow of filtrate, or whether reflux is aided by cessation or even reversal of filtration cannot be suggested from the present results. A remotely similar lesion was described by Bywaters and Dible[12] in their studies of the kidneys of patients with traumatic anuria. The lesion in that instance was noted 4 days following the onset of anuria and differed from the reflux lesion in that the "proximal" epithelial cells were attached to the basement membrane of Bowman's capsule. In occasional control rats an isolated glomerulus was seen in which Bowman's capsule was lined by proximal tubule epithelium. The sporadic occurrence of this phenomenon makes it unlikely that the reflux lesion could have been derived from cells normally lining Bowman's capsule since reflux was easily found in very many glomeruli in the experimental animals.

The occurrence of necrosis and reflux in the infra-glomerular segment of the proximal tubule need not indicate any unusual susceptibility of this portion of the nephron to the ischemic effects of serotonin-induced vasospasm. Indeed, the necrobiotic changes in the affected cells could not be distinguished from those in other parts of the proximal tubule in which cellular displacement was not noted. It seems more likely that the displacement of the dead cells occurred simply because there was a space available into which they could be forced. The precise nature of the propelling force or forces may well be more complex than suggested above. The morphologic evidence simply indicates the direction of propulsion.

Protein was commonly found in the Bowman's spaces of animals killed 3 hours after serotonin injection. In some instances, the precipitate was in the form of a homogeneous deposit while in others it was rather coarsely flocculent. The two types of precipitate not infrequently coexisted. The homogeneous deposit was indistinguishable from that seen in human kidneys with glomerular disease and clinical proteinuria. The flocculent precipitate, however, appeared identical to that found in lower segments of the nephrons in serotonin-treated rats. In many instances this appeared to be derived, at least in part, from tubular epithelial cytoplasmic breakdown. It closely resembled the protein seen in glomerular capillaries by Sheehan and Davis[13] in rabbit kidneys with "failed reflow" after prolonged renal ischemia. It is concluded, therefore, that the flocculent protein in Bowman's spaces in our animals was probably largely derived from the breakdown of tubule epithelial cyto-

plasm, and represented a deposit similar to the "intracapillary microfoam" described by Sheehan and Davis.[13] It might be further suggested that all or part of the flocculent protein so frequently found in Bowman's space in acute renal failure in man may have come up the nephron from lower down rather than being a reflection of otherwise invisible glomerular damage, as is usually suggested.

In the further evolution of tubule damage, pyknosis of nuclei was rapidly followed by fragmentation or extrusion of nuclei or nuclear fragments as basophilic droplets into the lumens. During the first 24 hours, both cytoplasmic and nuclear debris were found scattered diffusely throughout the cortex. Thereafter, however, the basophilic droplets and cytoplasmic fragments were concentrated just inside the corticomedullary junction. This localization of larger particulate debris probably represented an arrest of cellular fragments at the point of narrowing in the thin limb of Henle's loop, or possibly at the point of slight constriction where the distal convoluted tubule entered a collecting duct.[14] The coincident development of dilatation of tubules and epithelial regeneration has been noted by various authors.[1,2,9–11,15] Oliver[10] concluded that obstruction of tubule lumens was probably the primary cause of tubular dilatation, and thus interpreted it as a phenomenon of overdistention. Fiore-Donati and Erspamer[2] considered various causes, among them mechanical obstruction, compression of tubules by interstitial edema, obstruction by abundant necrotic epithelium, and hyperfunction of surviving nephrons. While it is possible, and even probable, that all of these factors were concerned in the production of dilatation, it was noteworthy in the present experiments that dilatation persisted, though in diminishing amounts, up to the third week after serotonin insult. This persistence of dilatation after the phenomena of healing were virtually complete would be in keeping with the earlier suggestion[1] that at least part of the distention resulted from immaturity of the newly regenerated epithelium and the inability of these young cells to cope with any considerable volume of glomerular filtrate. In man, following episodes of acute renal failure, inability to form concentrated urine may persist for 6 months or longer.[8,16,17] Gowing and Dexter,[18] in an investigation of recovery of enzyme activity following one hour of renal ischemia in rats, found that maximum recovery was not reached until 40 days, and that tubule epithelium in scarred areas appeared to regain its enzyme complement imperfectly, even after 133 days following a 1-hour ischemic episode.

In the later stages of recovery after serotonin-induced ischemic damage, there was progressively increasing interstitial cortical scarring. This was manifest as peritubular collagen deposition and moderate to

pronounced fibrosis surrounding Bowman's capsules. The phenomenon of pericapsular scarring with preservation of intact glomeruli has been commonly cited [19] as a change characteristic of chronic pyelonephritis. The regular appearance of the phenomenon in the healed stages of ischemic damage would appear to warrant the conclusion that, in these circumstances at least, it is more probably related to transient ischemia.

Sheehan and Davis [20] demonstrated that the sensitivity of the proximal tubule epithelium to transient ischemia may be greatly reduced under the effects of prolonged ether anesthesia. This they related to a better renal blood reflow in anesthetized than in unanesthetized animals. In contrast, de Wardener [21] concluded that nearly all anesthetic agents caused a reduction of renal blood flow and that this reduction was greater in deep than in light anesthesia. In the present experiments the effects of anesthesia *per se* were not studied, but in the animals subjected to light ether narcosis, lesions were different neither in frequency nor severity from those of control animals managed without anesthesia.

In an earlier study [1] it was found that the lethal effects of serotonin on pregnant rats were far greater than in the nongravid, a finding substantiated by Poulson, Botros and Robson.[5] The latter investigators interpreted their findings to be in keeping with the suggestion that serotonin may, in fact, be of considerable importance in the pathogenesis of the toxemia of pregnancy. Although there is no direct evidence on this point in the foregoing data, it is noteworthy that the nephropathic effects of serotonin appeared more intense in animals that were sick or traumatized. Thus, of 8 such animals in our series of experiments, 3 had renal lesions graded as IV, 1 as grade III, 3 as grade II, and only 1 had the minor grade I severity of renal damage.[1] This striking increase in severity of renal lesions in ailing animals may well reflect a nonspecific enhancement of serotonin toxicity in various forms of illness, among which pregnancy might possibly be included.

Summary

The most striking early manifestation of renal damage resulting from serotonin-induced ischemia was a reflux of the epithelium in the infra-glomerular segment of the proximal convoluted tubule into Bowman's space. The lesion was transient and was not found later than 12 hours after serotonin injection. Epithelial reflux appeared to result from the rapid development of both necrosis and detachment of epithelial cells and their upward displacement due to edema of more distally placed tubule epithelium. In the subsequent evolution of the renal lesions there was first a sludging of cellular debris in the region of the corticomedullary junction. This was followed by rapid epithelial regeneration con-

current with pronounced tubule dilatation. Three weeks after serotonin injection, there was still residual tubule dilatation accompanied by interstitial and pericapsular fibrosis of Bowman's capsule. It is considered that pericapsular fibrosis, in this instance at least, was a manifestation of ischemic damage rather than of pyelonephritis as conventionally interpreted.

Serotonin nephropathy appeared to be more severe in animals sick or traumatized at the time of injection. The possible relation of this finding to that of increased susceptibility of pregnant animals to serotonin is considered.

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LEGENDS FOR FIGURES

Except where indicated, sections photographed were stained with hemalum-phloxine-saffron.

FIG. 1. Reflux of proximal tubule epithelium into Bowman's space with compression of adjacent glomeruli in a rat killed 2½ hours after serotonin injection. Nuclear pyknosis and increased cytoplasmic density are apparent in the affected epithelium. × 244.

FIG. 2. An epithelial reflux lesion with pronounced basal edema in proximal tubule epithelium 3 hours after serotonin injection. Periodic acid-Schiff (PAS) stain. × 242.

FIG. 3. A subsiding reflux lesion 12 hours after serotonin injection. Note the complete loss of nuclear staining in the infra-glomerular segment of the proximal tubule. × 184.

FIG. 4. Coarse flocculent protein debris in the lumen of proximal tubules and in Bowman's space 3 hours after serotonin injection. Nuclear pyknosis in tubule epithelium is striking. × 184.

FIG. 5. A vacuolated homogeneous protein in Bowman's space 3 hours after serotonin injection. Note both the homogeneous and flocculent protein in the lumen of tubules lined by flattened epithelium, indicating loss of cytoplasmic substance. × 102.

FIG. 6. Basophilic droplets derived from extruded pyknotic nuclei lie in the lumens of tubules 3 hours after serotonin. × 77.

FIG. 7. Twenty-four hours after serotonin injection, there is complete loss of nuclear staining in necrotic tubules whose lumens are now filled by fragmented, dead epithelial cells. Note also the relative preservation of tubules surrounding a vein in the upper center. × 36.

FIG. 8. Twenty-four hours after serotonin injection, active mitotic regeneration of tubule epithelium appears. A necrotic cell with pyknotic nucleus is in the process of extrusion into the lumen of the tubule in the center of the picture. × 410.

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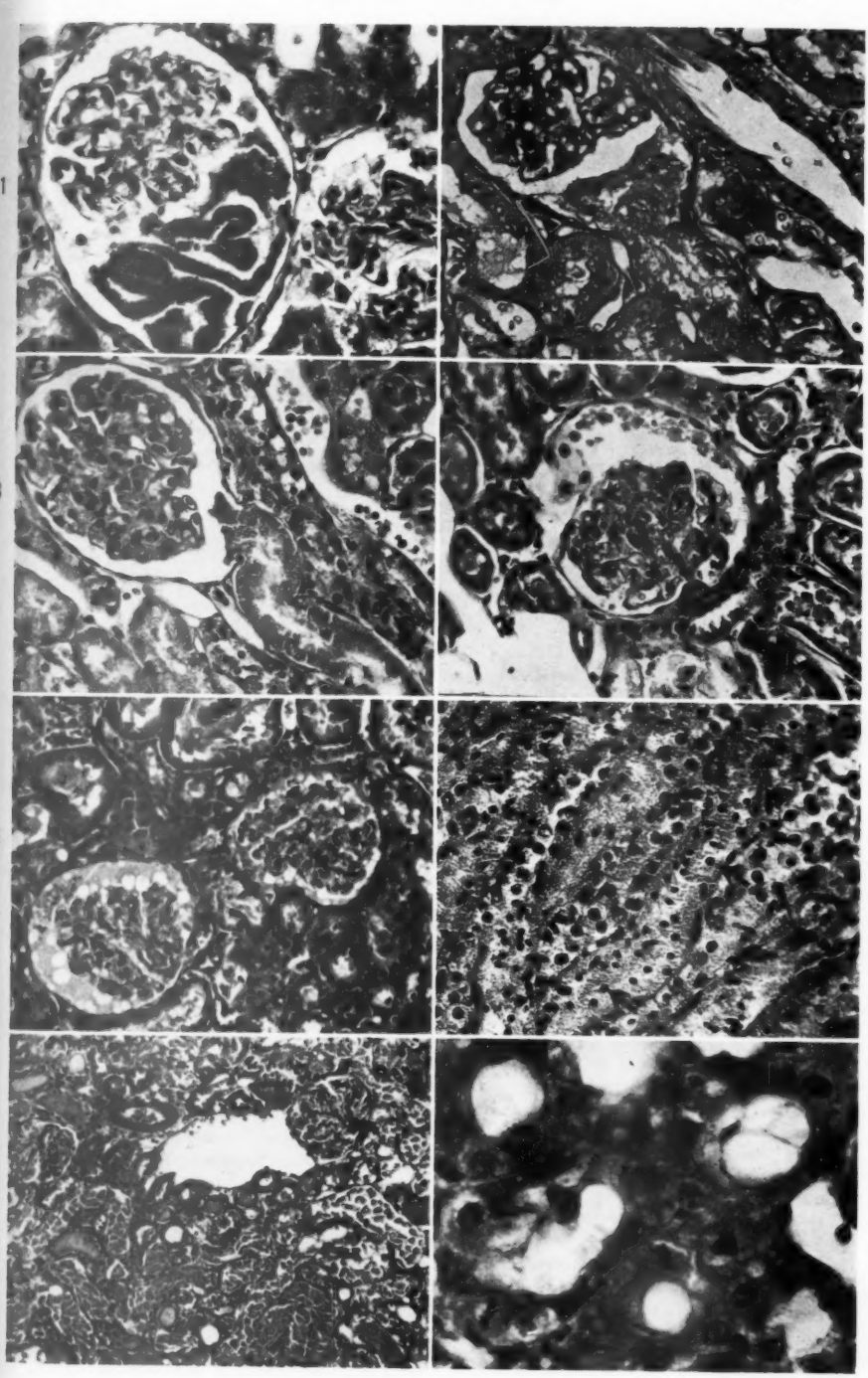
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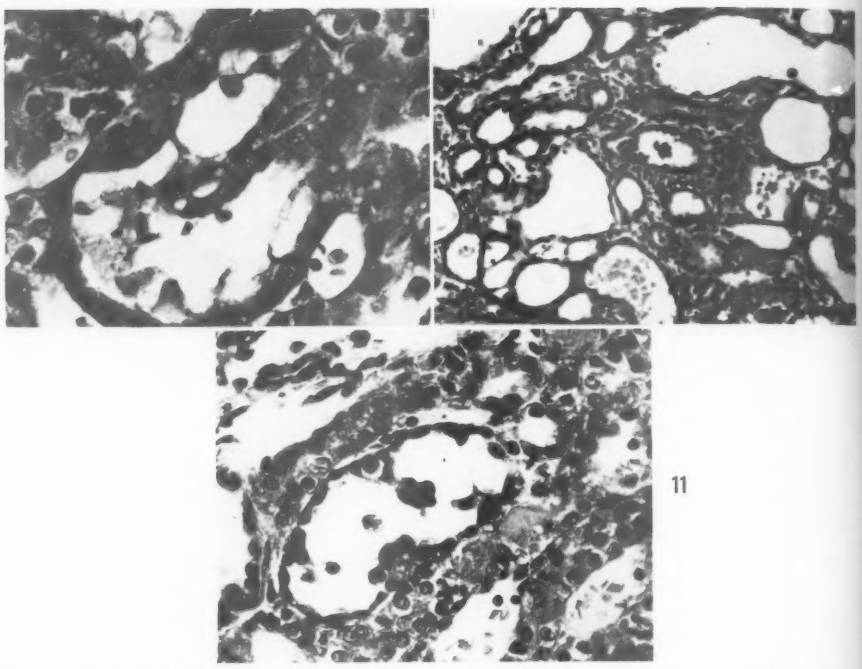
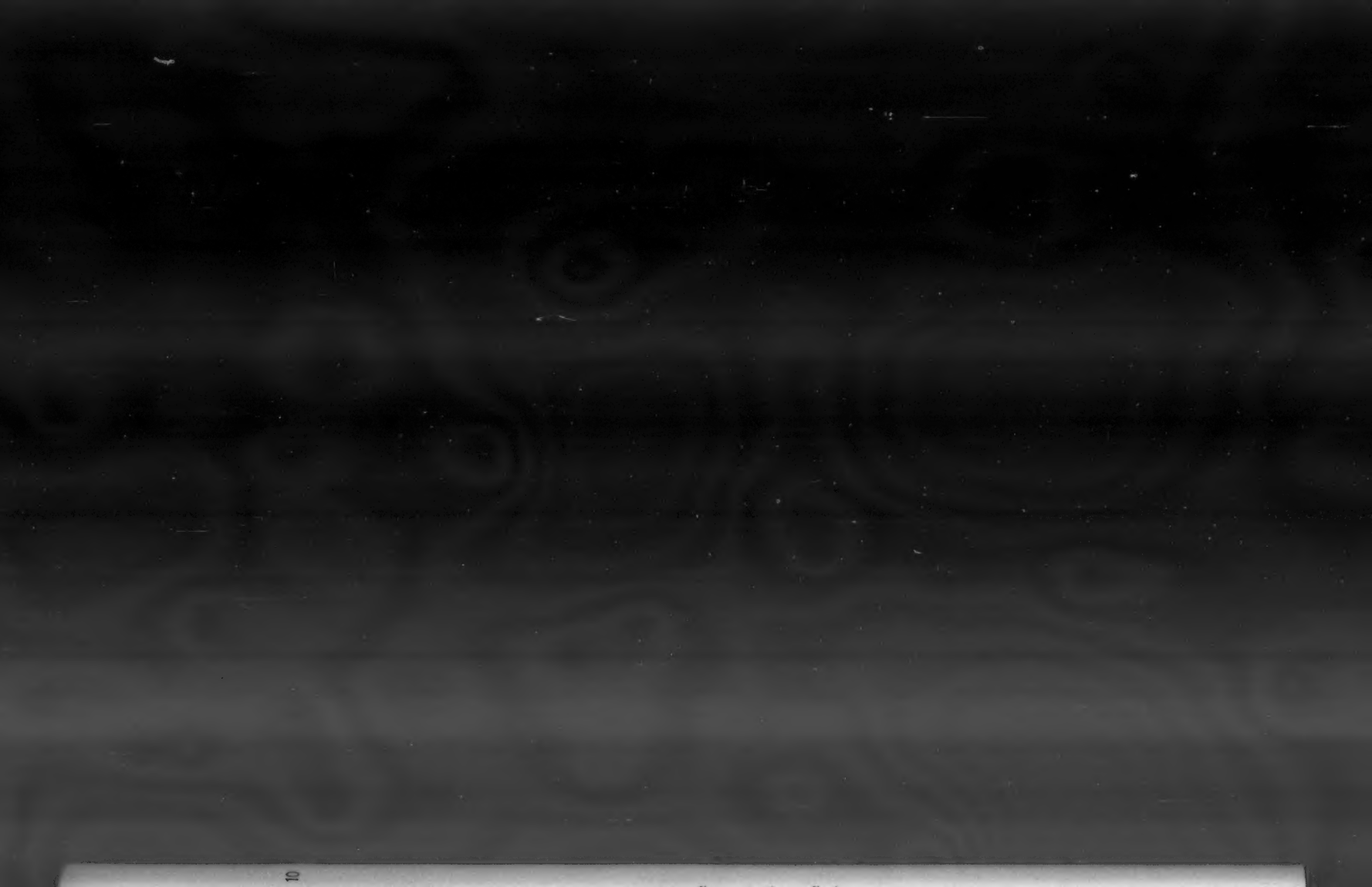


FIG. 9. Two days after serotonin injection, bizarre mitosis in a proximal convoluted tubule may be seen. × 345.6.

FIG. 10. Six days after serotonin injection, there is pronounced dilatation of tubules that are now lined by markedly flattened epithelium and relatively clear of intraluminal debris. × 244.

FIG. 11. Four days after serotonin injection, regenerative polyps protrude into tubule lumen. Note also close packing of nuclei along the basement membrane and scanty cytoplasm of regenerating cells in the center tubule. × 244.



REGENERATION OF THE LIVER

Absence of a "Humoral Factor" Affecting Hepatic Regeneration in Parabiotic Rats

Adrianne E. Rogers, M.D.*; James A. Shaka, M.D.;
Giselle Pechet, M.D., and Richard A. MacDonald, M.D.

From the Mallory Institute of Pathology, Boston City Hospital, and the Department of Pathology, Harvard Medical School, Boston, Mass.

The mechanism by which regeneration of the liver is initiated and controlled has long been an important but unsolved problem in biology and medicine. Three facets into which hepatic regeneration might be subdivided for purposes of investigation are: (a) its initiation following partial hepatectomy or injury; (b) its modification, once begun, as by hormones, nutrients, and possibly blood flow; and (c) its termination when the organ has regained its original size or function. The present work is concerned primarily with elucidating the factor or factors that initiate the regenerative process, although all 3 aspects are probably closely interrelated.

The reports by Christensen and Jacobsen,[1] Bucher, Scott and Aub,[2] and Wenneker and Sussman[3] of a stimulus to mitosis in the liver of one parabiotic rat as the result of partial hepatectomy in its partner directed attention to the possibility that hepatic regeneration might be controlled by an alteration in the composition of the blood, a so-called "humoral factor." Many studies of the control of regeneration followed this work. These included the injection of serum and liver homogenates from partially hepatectomized rats into normal rats, measurements of chemical constituents of the blood, effects of serum on tissue culture growth of cells, and investigation of the organ specificity of the stimulus to hepatic regeneration. As reviewed elsewhere,[4] the findings and conclusions in these investigations were conflicting. Consequently, the evidence that a humoral factor is involved in regeneration of the liver rests upon the original observations in parabiotic rats.

The 3 studies in parabiosis cited indicated that a stimulus to hepatic regeneration was detected in a "nonoperated" † partner by quantitation

Aided by grants H-3647, C-4666, and C-5313 from the United States Public Health Service, in part by a grant from the Massachusetts Division of the American Cancer Society, and in part by Contract DA-49-007-MD-863, Office of the Surgeon General, United States Army.

Accepted for publication, May 23, 1961.

* Research fellow, Harvard Medical School.

† The term "nonoperated" in referring to parabiotic rats is used to mean that no operative experimental procedure was done after parabiotic union was established.

of mitoses in its liver. In none of the 3 studies was there an indication that cross circulation between the parabiotic animals had been demonstrated, although it may be assumed that it was present. Christensen and Jacobsen used 3 sets of parabiotic paired rats, without parabiotic controls; they found hepatic mitoses to be slightly more numerous in the 3 nonoperated partners than in single nonoperated rats.[1] Wenneker and Sussman used 8 pairs of parabiotic rats without either nonoperated or sham operated control parabiotic animals.[3] They sacrificed 1 or 2 pairs at 2, 3, 5, 8 and 14 days after partial hepatectomy in one member. Counts of hepatic mitoses in the nonoperated members were compared with those in nonoperated single rats and were found to be slightly greater at 2 to 8 days after operation.

Bucher and co-workers used 22 pairs of parabiotic rats in 3 experiments at 24, 48 and 72 hours after partial hepatectomy and 3 sets of "triplet" parabiotics at 48 hours after partial hepatectomy.[2] As controls, mitosis counts were made in the liver portions removed at operation; no separate nonoperated or sham operated parabiotic rats were studied. In one of the experiments using paired rats, the difference between the number of hepatic cell mitoses in the nonoperated partners and in the control livers was not of statistical significance when all rats were used for calculations. The authors did not use some rats for statistical computation because on histologic examination cross circulation was not considered to have been established. In the other 2 groups the differences were of statistical significance. Colchicine enhanced none of the differences. In the parabiotic triplets, partial hepatectomy was performed in 2 members; here there was an even greater mitotic count in the liver of the nonoperated rat. Other indexes of regeneration investigated were the hepatic content of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), alkaline phosphatase, and total nitrogen, but these gave no evidence of a regenerative response.

A further study of parabiotic rats, carried out since the above reports, failed to confirm the earlier results. Islami, Pack and Hubbard[5] used 5 parabiotic pairs, with the liver removed at operation as the control. Their data showed no evidence, as indicated by mitoses, of a stimulus to regeneration in the liver of the nonoperated partner. It was suggested, however, that there was a depression of the regenerative response in partially hepatectomized parabiotic rats as the result of union with nonoperated rats.

The present investigation was undertaken after studies in which plasma from cirrhotic rats, rats with fatty liver, and those that had undergone partial hepatectomy was infused into normal rats.[4] The purpose of the preliminary investigations was to determine whether the

stimulus to increased mitosis and DNA synthesis observed in the livers of the 3 groups of donor rats [6] could be transmitted to the recipients. It was assumed from published reports that there would be a humoral stimulus to regeneration in the partially hepatectomized rats. No response in DNA synthesis or mitosis was found in any of the recipients, however. This was the case in animals that received plasma from partially hepatectomized rats. Moreover, the infusion of plasma from normal rats into animals that had undergone partial hepatectomy failed to inhibit regeneration. Therefore, it was considered necessary to restudy the existence of a humoral factor affecting regeneration of the liver in parabiotic rats.

Material and Methods

A total of 494 male Sprague-Dawley and 64 male inbred rats were used in these experiments. They were housed in an air-conditioned room and fed Purina Lab Chow and tap water *ad libitum*. Sprague-Dawley rats were purchased from the Charles River Breeding Laboratory, Boston, at 21 days of age, and inbred rats of the WR strain were purchased from the Endocrine Laboratories, Madison, Wisconsin, as weanlings. Both strains were maintained in this laboratory until used.

Sprague-Dawley rats (214; 107 pairs) were joined in parabiotic union by the open coelomic method [7]; 42 (21 pairs) were joined by the closed coelomic method.[8] Sprague-Dawley rats (174; 58 sets) and 57 inbred rats (19 sets) were joined as "triplets" by the open coelomic method. The skin incision extended from shoulder to hip joint; the peritoneal incision extended from the rib cage caudally for about 3 cm. Adjacent fore and hind limbs of partners were fixed by placing wire sutures through the proximal bones. The ages at the time of union varied from 36 to 78 days, and all the Sprague-Dawley parabiotic rats except some of the group of pairs examined at 72 hours after operation were composed of litter mates. The latter group contained a few non-litter mates; this had no detectable effect on the parabiosis or the experimental results. In addition, when single rats were used as controls, the single rats were litter mates of the parabiotic animals whenever possible, or were from the same lot.

The presence of cross circulation was determined by prompt (within 20 minutes), grossly visible excretion of phenolsulfonphthalein dye (PSP) in the urine of both members of pairs after injection of 0.5 ml. of the dye intramuscularly into one partner, or its excretion in the urine of all 3 members of triplets after injection of 1 ml. into one partner. Before the experimental procedures were performed, 6 pairs and 1 set of triplets that failed to show cross circulation and 7 pairs and 2 sets of triplets that appeared sick or runted were discarded. Mortality following parabiotic union and before use in experiments was 39 per cent among the pairs, 16 per cent among Sprague-Dawley triplets, and 58 per cent among the inbred triplets. Most deaths occurred within 3 weeks after parabiotic union. After partial hepatectomy or sham operation, there was an additional mortality of 20 per cent in the pairs, 35 per cent among the Sprague-Dawley triplets, and 20 per cent among the inbred triplets. Death was not attributable to any specific cause, and occurred following both sham operation and partial hepatectomy. Two parabiotic triplet rats exhibited such extensive and diffuse intrahepatic inflammation that they were discarded. One was a nonoperated partner of partially hepatectomized rats and one had undergone sham operation. Exclusion of their mitotic and autoradiographic counts did not affect the statistical significance of the conclusions in their groups.

As the result of the mortality and the discard of unsuitable animals, the experi-

ments were carried out using 53 pairs, 31 sets of Sprague-Dawley triplets and 7 sets of inbred triplets. Studies were made 24, 48, 72, and 96 hours after partial hepatectomy or sham operation. Nonoperated parabiotic animals were included to determine the effect of parabiosis alone and to study biologic variations in hepatic mitosis and DNA synthesis. The duration of parabiosis in the animals used was 7 to 46 days. In addition to parabiotic rats, 60 single Sprague-Dawley rats and 6 inbred rats were used as controls; 28 of the Sprague-Dawley animals underwent partial hepatectomy, 27 underwent sham operation; and 5 Sprague-Dawley and 6 inbred rats were nonoperated controls.

Partial hepatectomy, in which 61 to 80 per cent of the liver was removed, was performed in a standard manner under ether anesthesia. A double ligature was placed around the anterior and left lateral lobes, followed by excision of these lobes.[9] Sham operation consisted of a laparotomy with manipulation of the liver. Four hours before sacrifice tritiated thymidine (H^3-thymidine) of specific activity 1.9 curies per mM was injected intraperitoneally in divided amounts within less than 10 minutes. The dose was one μc. per gm. of body weight in all rats except the triplets examined 24 hours after operation. These received a dose of 0.5 μc. per gm. of body weight. The animals were killed by ether and necropsied immediately; the liver was weighed and slices were fixed in 10 per cent neutral buffered formalin.

Paraffin sections cut at 6 to 10 μ were stained with hematoxylin and eosin for mitosis counts. Stripping film autoradiographs stained with hematoxylin were prepared as described elsewhere[10]; exposure time of the films was 4 weeks. Mitotic figures, from late prophase through anaphase, were counted in 50 consecutive fields at a magnification of 560 times. Mitotic figures were noted frequently in Kupffer cells in parabiotic rats. These were counted and were present to the same extent in all groups except for an expected increase following partial hepatectomy; thus they were not considered further.

Hepatic nuclei labeled with H^3-thymidine were counted in autoradiographs in 100 consecutive fields at a magnification of 560; fields containing large portal areas and large blood vessels were positioned so that the connective tissue, bile ducts and vessels were omitted. The number of hepatic cells in a field (560 ×) was determined by using an eyepiece grid marker. The results were expressed per 100,000 hepatic cells. In livers with no mitotic figures or H^3-thymidine-labeled cells in the fields counted, a total of 200 fields was examined before giving zero as the result.

In Text-figures 1 to 7 all rats indicated were of the Sprague-Dawley strain unless otherwise noted. Statements of statistical significance refer to the t test at the 5 per cent level of confidence.

Results

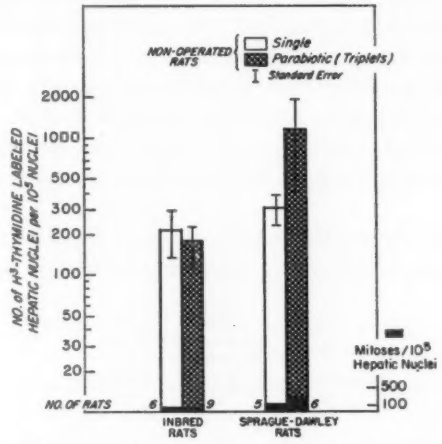
Histologic Features

The livers in both Sprague-Dawley and inbred parabiotic rats exhibited two findings which distinguished them from single animals. Plasma cells were present in the sinusoids as single cells or as clusters of 2 to 3 cells in 83 per cent of parabiotic rats, and small "granuloma-like" formations, composed of degenerating liver cells with an infiltrate of mononuclear cells and plasma cells occurred in 15 per cent. In addition, there were vascular lesions consisting of slight mononuclear cell infiltration in and around the walls of portal veins in two rats and in the adventitia of small hepatic arteries in a third. The livers were otherwise normal except in the two rats mentioned previously.

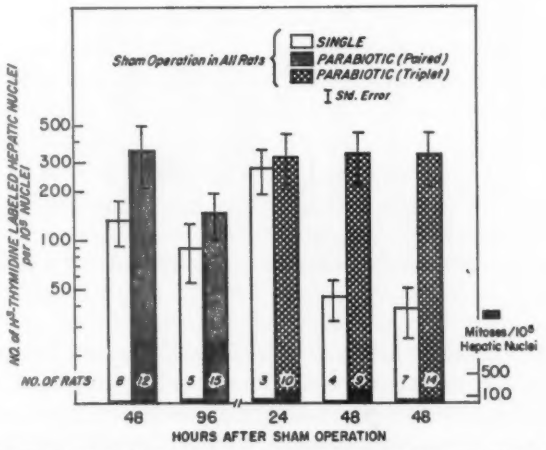
Autoradiographic and Mitosis Counts (Figs. 1 through 7)

In assessing other differences between parabiotic and nonparabiotic rats, it was observed that hepatic mitoses were more numerous and DNA was slightly greater in nonoperated parabiotic triplet rats than in nonoperated individual rats although the differences were not statistically significant (Text-fig. 1).

A difference in response to sham operation in parabiotic paired and



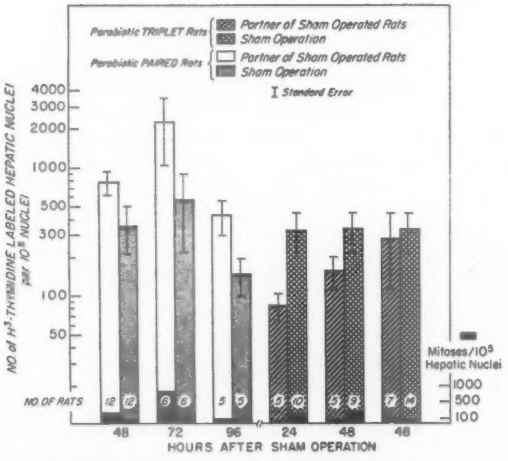
TEXT-FIGURE 1. DNA synthesis and mitosis in livers of nonoperated single compared with nonoperated parabiotic triplet rats.



TEXT-FIGURE 2. Effect of sham operation on DNA synthesis and mitosis in livers of single compared with parabiotic rats.

triplet rats as compared with individual rats was observed: hepatic DNA was consistently greater in parabiotic animals. In two separate experiments, studies were made 48 hours after a sham operation; triplet rats of the Sprague-Dawley strain showed significantly more DNA synthesis than their single sham operated controls. In one of the experiments mitotic activity was also significantly greater (Text-fig. 2). In addition, there was a variable response in different groups of animals. When individual rats were subjected to sham operation, their livers showed less DNA synthesis and mitotic figures 48 hours later than their nonoperated controls. In paired animals, the members that underwent sham operation had less hepatic DNA synthesis and fewer mitotic figures 48, 72, and 96 hours later than their nonoperated partners. In triplet parabiotic rats, where two lateral members underwent sham operation, the operated rats, 24 and 48 hours after the operations, showed no consistent differences from their nonoperated partners or from nonoperated sets of triplets.

These findings suggest that sham operation depressed hepatic DNA synthesis and mitotic activity in single and parabiotic paired rats, but

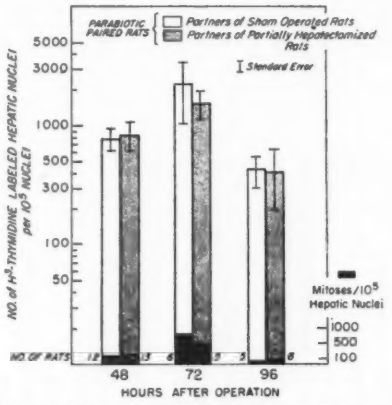


TEXT-FIGURE 3. Effect of sham operation on DNA synthesis and mitosis in livers of parabiotic rats.

did not do so in triplet rats. In addition to its effect in an operated parabiotic rat, a sham operation in one member may have stimulated hepatic DNA synthesis and mitotic division in the nonoperated partner. At 48 and 72 hours after operation, mitotic figures were significantly more numerous in a nonoperated rat than in its operated partner, and

more numerous, but not significantly so, than in parabiotic pairs where both rats were nonoperated. Hepatic DNA synthesis was similarly greater, but not significantly so, in the nonoperated partners. These findings were consistently observed in pairs, but were not noted in triplets (Text-fig. 3).

The effect of partial hepatectomy on hepatic DNA synthesis or



TEXT-FIGURE 4. Lack of effect of partial hepatectomy in one parabiotic rat on DNA synthesis and mitosis in liver of nonoperated partner.

mitotic activity in one member of paired rats was no greater than sham operation alone in the livers of nonoperated partners at 48, 72 and 96 hours (Text-fig. 4). Partial hepatectomy in 2 lateral members of triplets also caused no statistically significant elevation of hepatic DNA synthesis or mitosis in nonoperated partners 24 or 48 hours after operation. Variation in hepatic DNA synthesis and mitosis in the parabiotic rat was such that nonoperated Sprague-Dawley triplets showed higher average values than partners of operated rats (Text-fig. 5).

This would indicate that in neither paired nor triplet rats was there definite evidence of a humoral stimulus to hepatic regeneration (Table I). Sham operated inbred triplets were not included in these studies because their great mortality reduced the number available. From the observations in sham operated Sprague-Dawley triplets, hepatic DNA synthesis and mitosis would be expected to be the same as or greater than nonoperated controls.

Regeneration of the liver after partial hepatectomy in paired and triplet rats, although subject to variations in response, was similar to regeneration in single rats. There were no consistent differences at 24,

TABLE I

THE EFFECT OF PARTIAL HEPATECTOMY OR SHAM OPERATION IN PARABIOTIC RATS ON THEIR NONOPERATED PARTNERS

Hours between operation and sacrifice	No. of rats	Age at sacrifice (days)	Type of rat	Operation on parabiotic partners	Days between parabiosis and operation	H^3-thymidine labeled hepatic nuclei*	Hepatic mitoses*
Parabiotic pairs							
48	13	62 — 113	Sprague-Dawley	Partial hepatectomy	15 — 33	829 ± 219†	205 ± 68†
48	12	62 — 99	Sprague-Dawley	Sham	15 — 43	774 ± 162	192 ± 48
	7‡	78 — 113	Sprague-Dawley	None	19 — 46	313 ± 145	97 ± 22
72	5	61 — 103	Sprague-Dawley	Partial hepatectomy	15 — 31	1,551 ± 411	527 ± 126
72	6	54 — 94	Sprague-Dawley	Sham	10 — 28	2,244 ± 1,206	787 ± 194
	4	54 — 99	Sprague-Dawley	None	11 — 28	1,114 ± 516	558 ± 250
96	6	83 — 104	Sprague-Dawley	Partial hepatectomy	22 — 36	409 ± 213	95 ± 41
96	5	83 — 104	Sprague-Dawley	Sham	24 — 35	422 ± 130	60 ± 24
	7‡	78 — 113	Sprague-Dawley	None	19 — 46	313 ± 145	97 ± 22
Parabiotic triplets							
24	2	70	Sprague-Dawley	Partial hepatectomy	11 — 12	169 ± 99	16 ± 11
24	5	70	Sprague-Dawley	Sham	11 — 15	83 ± 20	32 ± 9
	3	70	Sprague-Dawley	None	11	92 ± 41	34 ± 14
48	5	75	Sprague-Dawley	Partial hepatectomy	8 — 21	818 ± 230	203 ± 110
48	7	75	Sprague-Dawley	Sham	7 — 22	281 ± 166	66 ± 26
	6	75	Sprague-Dawley	None	11 — 21	1,180 ± 766	291 ± 204
48	4	80 — 91	Inbred	Partial hepatectomy	10 — 17	1,053 ± 523	136 ± 61
	9	80 — 91	Inbred	None	14 — 18	166 ± 56	46 ± 18

* Expressed per 10^5 hepatic nuclei. H^3-thymidine labeled nuclei were counted in autoradiographs.

† Standard error.

‡ These represent the same rats; the 48 and 96 hour experiments were done simultaneously; one nonoperated group of controls was used for both.

TABLE II

REGENERATION OF THE LIVER FOLLOWING PARTIAL HEPATECTOMY IN PARABIOTIC AND SINGLE RATS*

Exper. no.	Hours after partial hepatectomy	No. of single rats	No. of parabiotic paired rats	No. of parabiotic triplet rats	Partial hepatectomy (%) †	H^3-thymidine labeled hepatic nuclei ‡	t test	Hepatic mitoses ‡	t test
1	48	4			76 ± 1§	10,060 ± 1,327§	$P < 0.05$	1,131 ± 157§	$P > 0.05$
			6		63 ± 4	5,508 ± 772		1,578 ± 288	
2	48	5			61 ± 4	7,596 ± 637	$P > 0.05$	1,283 ± 177	$P > 0.05$
			7		67 ± 3	5,946 ± 960		1,679 ± 319	
3	96	4			80 ± 2	1,596 ± 623	$P > 0.05$	284 ± 85	$P > 0.05$
			6		62 ± 7	1,863 ± 604		396 ± 161	
4	24	3			Not	31,539 ± 5,721	$P > 0.05$	5,279 ± 1,890	$P < 0.05$
				4	determined	13,897 ± 6,305		402 ± 133	
5	48	8			73 ± 3	8,010 ± 1,145	$P > 0.05$	3,375 ± 445	$P < 0.01$
				11	65 ± 3	6,199 ± 669		1,837 ± 183	

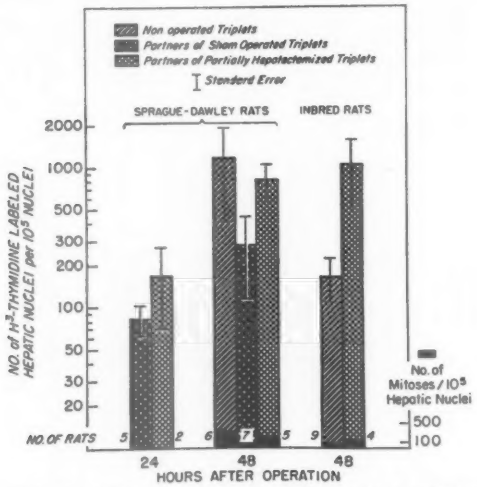
* A comparison with nonoperated parabiotic rats may be made in each experiment by referring to the appropriate group in Table I.

† Total liver weight at operation was calculated from body weight at sacrifice.

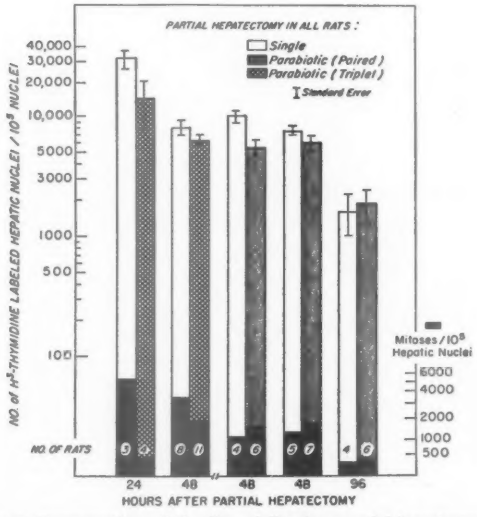
‡ Expressed per 10^8 hepatic nuclei. H^3-thymidine labeled nuclei were counted in autoradiographs.

§ Standard error.

48 and 96 hours (Text-fig. 6; Table II). The variation in response at any one time after partial hepatectomy was similar to that noted by us previously among groups of single rats.

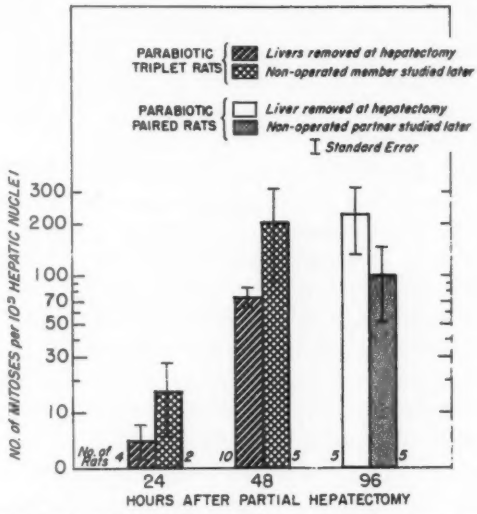


TEXT-FIGURE 5. Lack of effect of partial hepatectomies in 2 triplet parabiotic rats on hepatic DNA synthesis and mitosis in their nonoperated partners.



TEXT-FIGURE 6. Regeneration of the liver following partial hepatectomy in parabiotic and single rats.

Because other workers in parabiosis had done so, we compared mitotic activity in specimens removed at partial hepatectomy with that in the liver of nonoperated partners later, at time of sacrifice. The slightly increased amount of hepatic mitosis in the triplet rats whose partners had had partial hepatectomy was within the range of variation observed in these and other studies. The variation in mitosis is shown by comparing



TEXT-FIGURE 7. Lack of effect of partial hepatectomy in 1 or 2 parabiotic rats on hepatic mitosis in 1 nonoperated member.

the livers removed at partial hepatectomy in the 24 and 48 hour groups (Text-fig. 7). In the case of paired rats, livers obtained at sacrifice from nonoperated rats showed fewer mitoses than in the livers of their partners at the time partial hepatectomy had been done. Here also, evidence of a stimulatory humoral factor after partial hepatectomy was not manifest.

COMMENT

We have found no definite evidence for either a stimulating or an inhibiting humoral factor directly governing hepatic regeneration in several groups of parabiotic rats. Our findings therefore contrast with those of several earlier workers. There are a number of factors which may bear on the differences in our observations. The type of controls utilized is an important consideration. It is possible that parabiotic union itself may have an effect on an organ or parameter under investigation and that parabiotic animals may respond to a procedure differ-

ently from normal animals. For example, a difference in response between parabiotic and single rats is illustrated in Text-figure 2. Here it is shown that sham operated paired and triplet rats exhibited greater hepatic DNA synthesis than single controls. In addition, it was observed in paired rats whose partners had undergone a sham operation that there was significantly increased hepatic mitotic activity 48 and 72 hours after operation but insignificantly greater hepatic DNA synthesis.

Since a stimulating or inhibitory response may follow a sham operation, the reaction to partial removal of an organ must be measured against sham operated controls as well as other control groups, most importantly, nonoperated parabiotic subjects. In the studies previously cited this was not done.[1-3]

An explanation for what may have been a response of hepatic DNA synthesis and mitosis in a parabiotic rat whose partner had undergone a sham operation is not known. One possibility is the transmission between animals of a nonspecific protein material.[11] This is unlikely, however, because it was not noted in triplet parabiotic rats. Sham operation must be investigated further before any conclusion can be reached concerning its effect on cellular proliferation and on growth. In a study of parabiotic triplets not reported here in detail, a small (10 per cent) partial hepatectomy was done in the two lateral members. The findings were the same as with sham operation alone.

Other factors which may be of importance in interpretation are biologic variation, the existence of intraperitoneal or intrahepatic inflammation, or modifications of growth. There are normal variations in the indexes of metabolism and especially of growth. Thus, if two very small groups of animals are used, statistically significant differences may be found on occasion which are not related to the experimental procedure. This is particularly true in our experience in relation to the number of cells in mitosis at any one time in a rat liver. As was pointed out in an earlier publication,[4] small numbers of rats have been used in many studies of hepatic regeneration and in those involving the infusion of plasma or serum. Therefore, different conclusions may have been reached because of biologic variation.

Unpublished studies which we have made have shown that intraperitoneal or intrahepatic inflammation may result in stimulation, or alternating stimulation and depression of hepatic mitosis. Maximal hepatic DNA synthesis and mitotic activity in single rats following partial hepatectomy is known to occur approximately 24 hours after operation.[12] The transmitted response reported in some parabiotic rats, but which has not been found until 48 hours or more after operation, may have been due to increasing inflammation (or related phenomena) spreading into the connected peritoneal cavities.

Finally, consideration must be given to the effect of an operative procedure alone on the continuous process of growth and replacement of cells. Rats grow throughout their lives; a probable reflection of this, together with the factor of cell replacement, is the evidence of constant hepatic parenchymal DNA synthesis and mitosis.[13] These processes are continuous, but they wax and wane in intensity.[13] A wide variety of experimental procedures, especially those of stressful nature, may have a transient effect, especially on the process of mitosis, without leading to the continued growth or regeneration of an organ.[14] Manifestations which affect hepatic DNA synthesis or mitosis temporarily may not represent the same mechanisms that initiate and control hepatic growth and regeneration.

Summary

Regeneration of the liver was investigated in single and parabiotic paired and "triplet" rats following sham operation and partial hepatectomy. Quantitation of mitosis and DNA synthesis was determined with the aid of H^3-thymidine and use of autoradiographs.

Partial hepatectomy in one member of a parabiotic pair and in two members of parabiotic triplets failed to elicit a statistically significant response in hepatic DNA synthesis or mitosis in a nonoperated partner.

Hepatic DNA synthesis was greater in nonoperated parabiotic triplet rats than in nonoperated single rats, although differences were not of statistical significance.

After sham operation in parabiotic paired and triplet rats, greater hepatic DNA synthesis and mitotic activity was found than in single sham operated rats.

After a sham operation in a member of parabiotic paired rats, greater hepatic DNA synthesis and mitotic activity was found in the nonoperated partners.

Partial hepatectomy in parabiotic paired and triplet rats resulted in a regenerative response that was similar to that observed in partially hepatectomized single rats. Variations in the degree of response were found among different groups of parabiotic and single rats.

The present experiments do not support the conclusion that the blood in parabiotic rats carries stimulating or inhibitory "humoral factors" directly governing hepatic regeneration. This does not exclude the possibility that regeneration and growth may be modified by blood flow or by changes in the composition of the blood.

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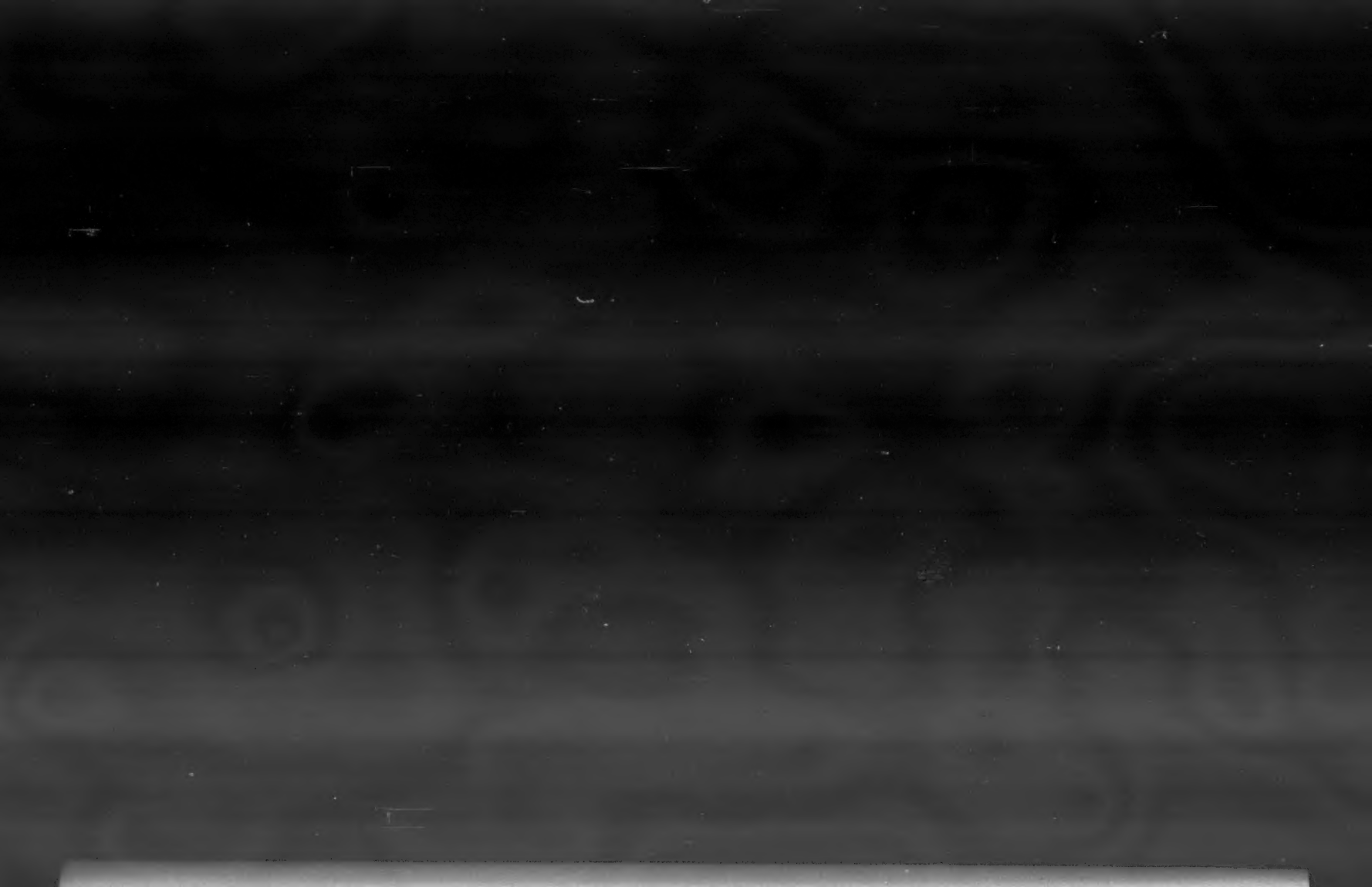
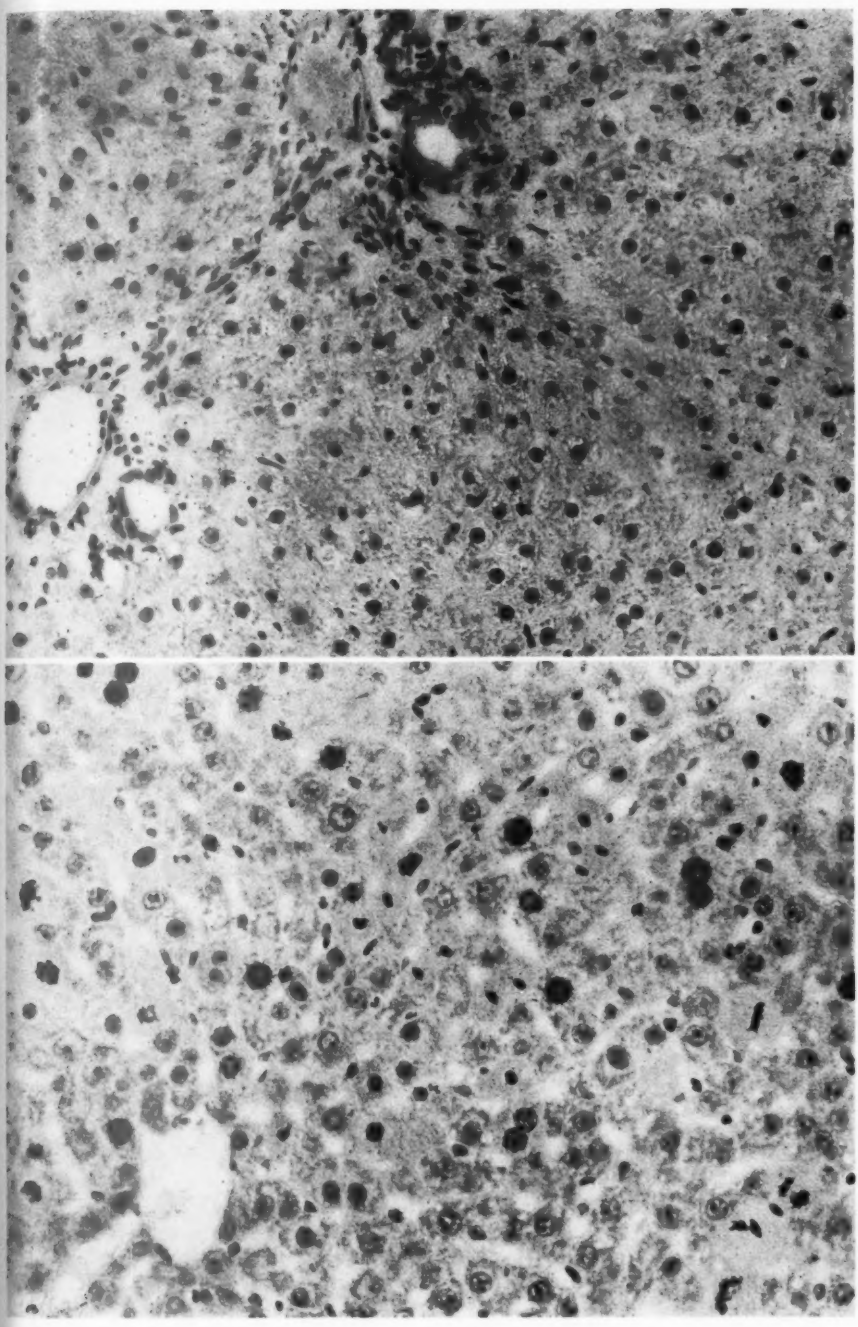
LEGENDS FOR FIGURES

All photomicrographs are autoradiographs of sections stained with hematoxylin.

FIG. 1. Liver of a normal, nonoperated, single rat that received 1 μc. of H^3-thymidine per gm. of body weight intraperitoneally 4 hours before sacrifice. There is one H^3-thymidine labeled hepatic cell nucleus. × 380.

FIG. 2. Liver of a single rat that underwent approximately 70 per cent partial hepatectomy and received 1 μc. of H^3-thymidine per gm. of body weight intraperitoneally 48 hours later, 4 hours before sacrifice. There are approximately 25 labeled hepatic nuclei, and several mitotic figures in hepatic cells. × 380.





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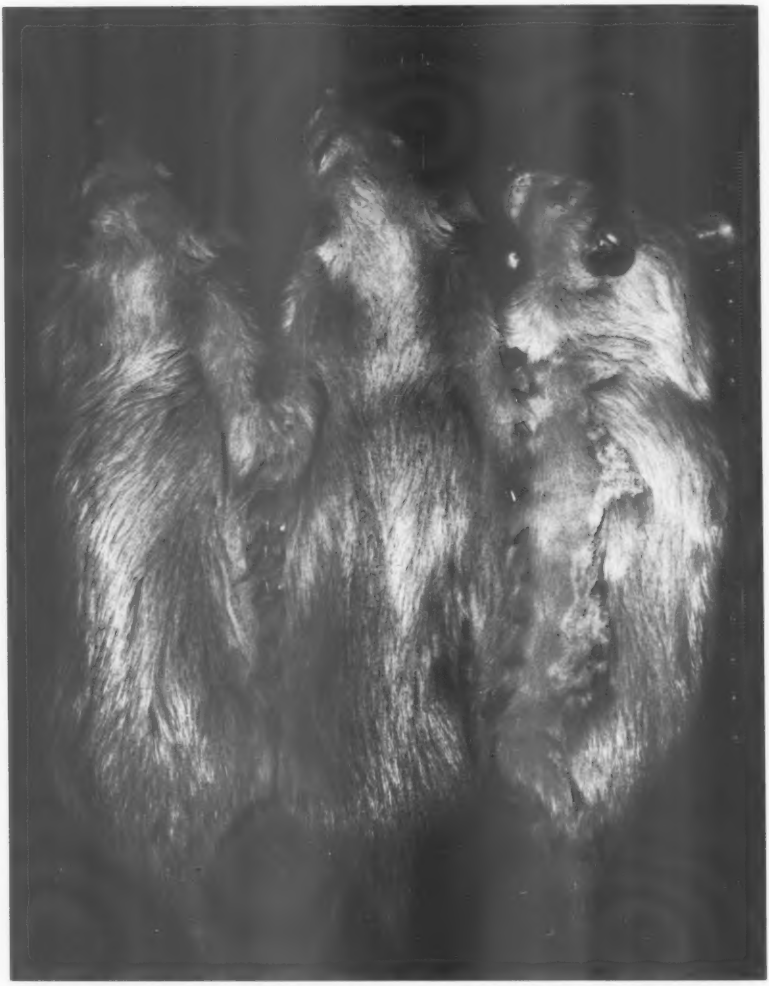
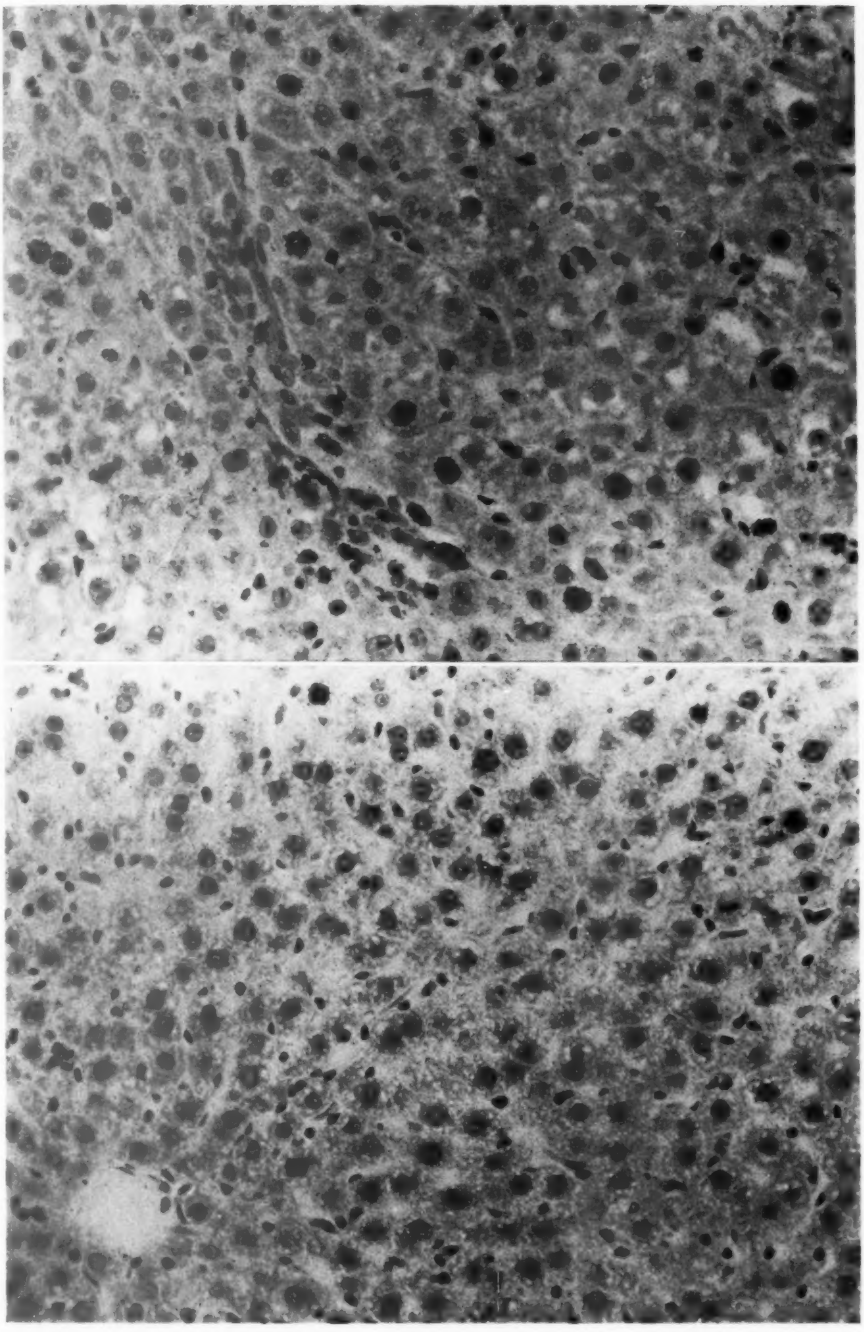


FIG. 3. Sprague-Dawley triplet parabiotic rats at the time of sacrifice. They are healthy, with abundant growth of hair along the lines of incision.

FIG. 4. Liver of a triplet parabiotic rat that underwent approximately 70 per cent partial hepatectomy and received 1 μc. of H^3-thymidine per gm. of body weight intraperitoneally 48 hours later, 4 hours before sacrifice. There are approximately 25 labeled hepatic cell nuclei, comparable to the number in the liver in Figure 2. × 380.

FIG. 5. Liver of a center, nonoperated triplet parabiotic rat whose two partners underwent approximately 60 per cent and 70 per cent partial hepatectomy. All 3 animals received 1 μc. of H^3-thymidine per gm. of body weight intraperitoneally 48 hours later, 4 hours before sacrifice. There are approximately 5 labeled hepatic nuclei and occasional mitotic figures in hepatic cells. × 380.



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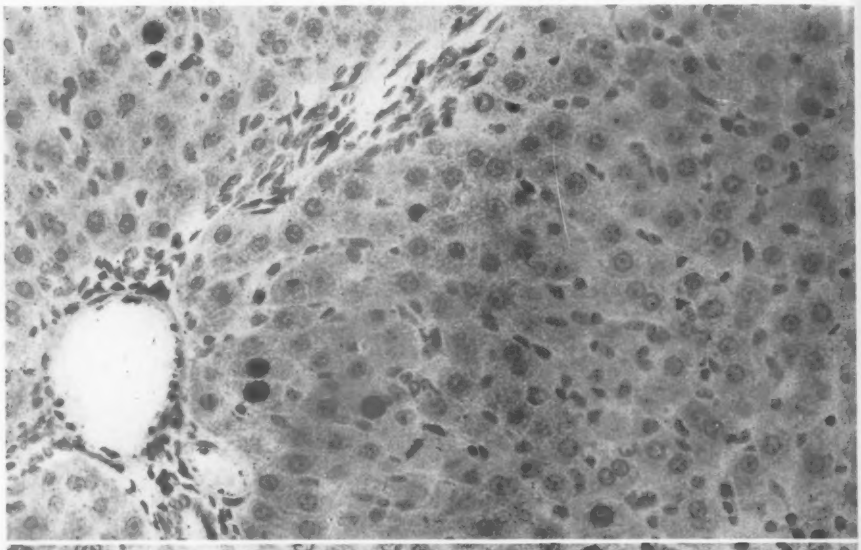

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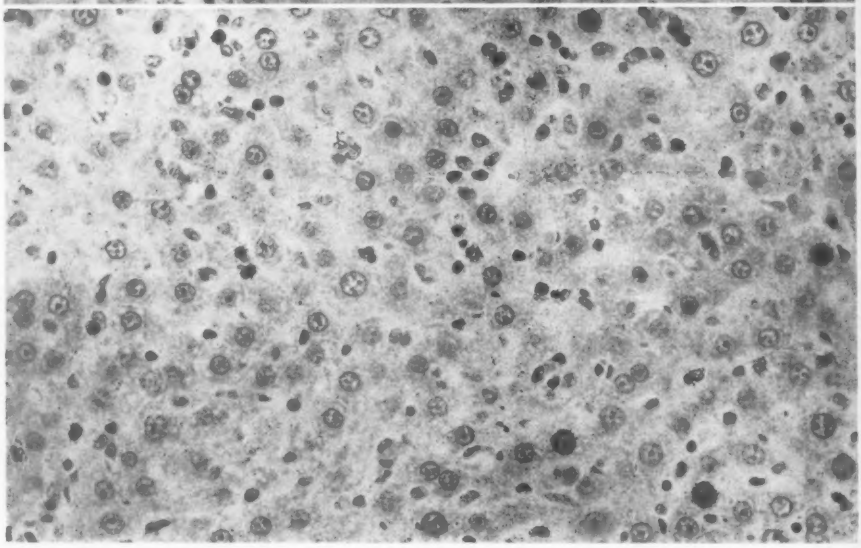
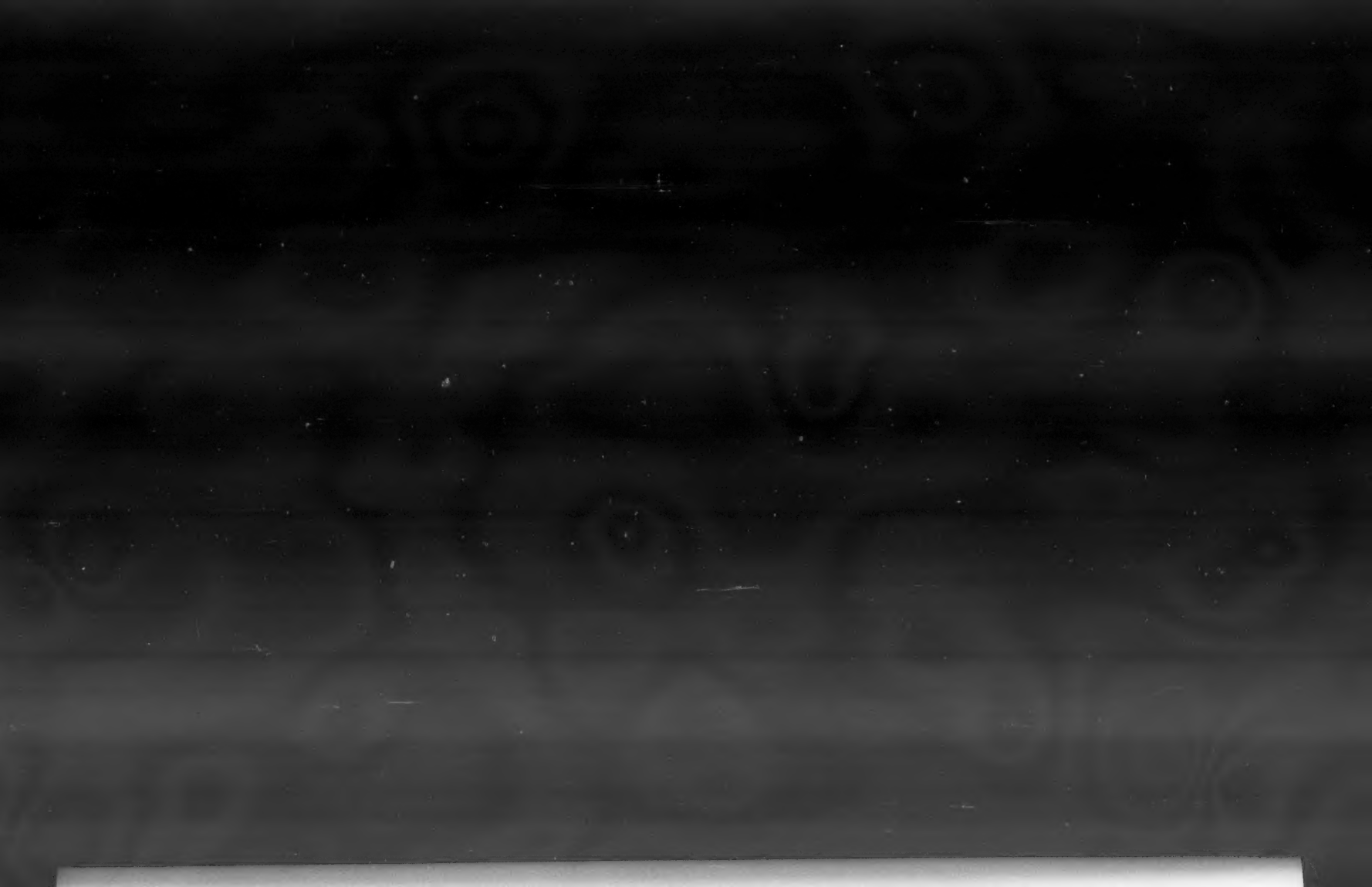


FIG. 6. Liver of a parabiotic rat from a triplet set in which no operation other than parabiosis was performed. All animals received 1 μc. of H^3-thymidine per gm. of body weight 4 hours before sacrifice. There are approximately 7 labeled hepatic nuclei, comparable to the number shown in the liver in Figure 5. This is the greatest amount of H^3-thymidine uptake noted in nonoperated parabiotic rats, and illustrates the considerable variation in DNA synthesis and mitosis encountered in parabiotic animals. × 380.

FIG. 7. Liver of a center, nonoperated triplet parabiotic rat whose two partners underwent sham operation. All 3 animals received 1 μc. of H^3-thymidine per gm. of body weight intraperitoneally 48 hours later, 4 hours before sacrifice. There are approximately 6 labeled hepatic nuclei, comparable to the number in the livers shown in Figures 5 and 6. × 380.



STUDIES ON THE RESISTANCE OF FOWL TO PNEUMOCOCCAL INFECTION

EDWARD C. ANDREWS, JR., M.D., AND GEORGE E. MCKINNON, M.D.*

From the Department of Pathology and Oncology, University of Vermont College of Medicine, Burlington, Vt., and the Department of Pathology, Johns Hopkins University School of Medicine, Baltimore, Md.

Since the time of Pasteur's original observation,[1] it has been assumed that domestic fowl are natively and therefore, by inference, genetically resistant to pneumococcal infection. This assumption, however, becomes questionable when the anti-pneumococcal properties of fowl serum are compared to those of acquired antibody. Bull and Bartual,[2] studying whole chicken blood *in vitro,* found opsonins and agglutinins for pneumococci. In subsequent experiments, Bull and McKee[3] reported that fowl serum would passively protect mice and guinea pigs against pneumococci and that this protective activity was type specific—i.e., it could be absorbed for one pneumococcal type without destroying the protective activity for other types. In these respects fowl serum is similar to the serum of animals immunized with pneumococci, and for this reason we questioned the old assumption that fowl are natively resistant. It seemed entirely possible that fowl could have had contact with pneumococci or other cross-reacting antigens early in life and that their resistance to pneumococcal infection could be due to acquired antibody. If this were the case, resistance to infection and serum protective activity might not be expected to appear until the birds were a few weeks old.

The experiments contained in this report were undertaken to determine when in the life of the chicken resistance to pneumococcal infection and serum protective activity appears, and to study, in adult fowl, the mechanism of this resistance *in vivo*. Resistance to infection and serum protective activity were found to develop shortly after hatching, and it appears that fowl do not destroy injected pneumococci by opsonification and phagocytosis. In these respects fowl differ from animals with acquired resistance to pneumococcal infection. Thus our evidence supports the assumption of a native or genetic origin for the resistance of fowl to pneumococcal infection.

Aided by Research Grant E-2334 from the National Institutes of Health, United States Public Health Service.

Accepted for publication, June 23, 1961.

* Present address: Charlotte Memorial Hospital, Charlotte, N.C.

Material and Methods

Animals

White Leghorn baby chicks of both sexes were purchased from a local hatchery. For most experiments, "dry," healthy chicks were selected from the incubators, taken to the laboratory, divided into groups and placed in brooders. Since eggs in any batch would have started hatching some 48 hours before removing the chicks from the incubators, their age, in hours, would vary considerably. In one series of experiments accurate and uniform age was insured by selecting chicks that had just broken their shells and were still "wet." The ages of "dry" chicks were estimated to be some 24 hours older than "wet" chicks. Careful selection and handling of "wet" chicks minimized spontaneous deaths. Chicks were given water and commercial chick mash *ad libitum*. The chick mash did not contain antibacterial drugs.

"Cross-bred" hens, 1 year old and weighing 2.5 to 3 kg., were maintained on a commercial mash not containing antibacterial drugs.

Young male Swiss albino mice, weighing 20 to 25 gm., were purchased from commercial suppliers and given pellets and water *ad libitum*.

Donor rabbits for normal serum were albino males weighing 2.5 to 3.5 kg.

Bacteria and Cultural Methods

Stock cultures of a type I pneumococcus, highly virulent for mice and rabbits, were prepared and stored as follows: The pneumococcus was passed serially through mice until highly virulent. A few drops of blood from the heart of an infected mouse were then cultured for 8 hours in trypticase soy broth. Several dozen 2 ml. vials containing 0.5 ml. of this broth culture and 0.5 ml. of sterile defibrinated rabbit blood were sealed with flame, quick frozen in a dry ice-alcohol bath and stored at −70° C. For each experiment, 5 ml. of trypticase soy broth was inoculated with 0.1 ml. of the material from a freshly thawed vial and incubated for 8 hours. This resulted in cultures containing approximately 10^9 pneumococci per ml. It was therefore possible to prepare, by appropriate dilution, inoculums for any given experiment which would contain the desired number of organisms. Since a large batch of frozen vials had been prepared from a single culture and all treated identically, except for the length of time stored at −70° C., the uniformity of virulence was also reasonably assured for each experiment. The number of organisms inoculated in each experiment was checked by counting colonies in pour plates and the virulence confirmed by the intraperitoneal inoculation of a few mice with 10^3 organisms.

Dead chicks and mice were bled aseptically from the heart and one drop placed in trypticase soy broth and one drop streaked on a blood agar pour plate. The plates were incubated for 24 hours at 37° C. Pneumococci were identified by colonial structure and microscopic examination of Gram stained smears.

The skin specimens of the chickens in experiment 3 that were to be cultured were removed aseptically, bisected, and one half cultured by inserting a sterile platinum wire directly into the injection site. It was then streaked on a blood agar pour plate. Several insertions and streaks were made on each specimen. The plates were incubated for 24 hours and pneumococci identified as before.

Serum

For passive protection tests, serum was collected and treated as follows: Baby chicks were exsanguinated by severing the jugular vein and carotid artery. For each experiment the blood was pooled from 50 or 60 chicks of the same age and from the same hatch. Blood from chickens 10 weeks old or older was collected at a local abattoir. Rabbits were bled by cardiac puncture. The blood from both chickens and rabbits was allowed to clot and stand at room temperature for 1 hour, left in the refrigerator overnight and centrifuged the following morning. The serum was har-

vested and cultured for sterility; if found contaminated (rarely) it was passed through a Seitz filter. If serum was to be held for more than 48 hours, it was stored at −20° or −70° C.

Inoculations

Baby chicks received 0.1 ml. of an 8-hour type I culture containing 10^8 organisms subcutaneously beneath the wing. In passive protection tests mice were given 2 ml. of the appropriate serum intraperitoneally, 4 to 6 hours before intraperitoneal infection with a saline dilution containing 10^3 pneumococci.

Specimens for Microscopic Study

Blocks of tissue were fixed in Zenker's formol solution and embedded in paraffin. Hematoxylin and eosin, Brown and Brenn, and Giemsa stains were made on sections cut at 5 μ.

Results

The Appearance of Resistance to Pneumococcal Infection in Baby Chicks

In determining whether species resistance to any infection is genetic or acquired, it is essential to know at what age the resistance appears. The following experiments were performed in order to determine the age at which resistance to pneumococcal infection appears in chickens.

Groups of chicks varying in age from 3 hours to 21 days were given 10^8 type I pneumococci subcutaneously. Chicks from the same hatch and of the same age were kept under identical conditions as controls. Spontaneous deaths among these birds were rare, and to further rule out the possibility of deaths from other causes, the heart blood of dead chicks was cultured. Representative chicks were necropsied and tissues taken for microscopic examination.

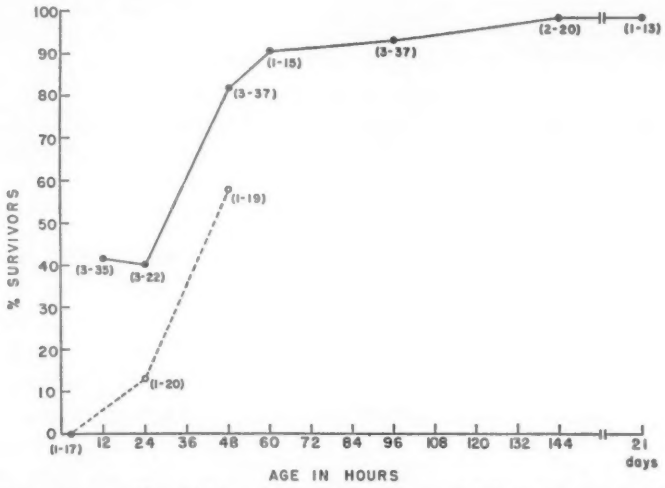
Freshly hatched chicks, still "wet" and less than 3 hours old, were uniformly killed by 10^8 pneumococci. Half of the chicks 48 hours old, nearly all of the chicks 4 to 5 days and all 21-day-old chicks survived a similar infection (Text-fig. 1). With rare exceptions, pneumococci were cultured from the heart blood of dead chicks. Microscopic sections of inoculation sites from dead chicks showed swarms of pneumococci with a few leukocytes and many red cells. Grossly these areas were edematous and inflamed, the process often extending down the leg. Sections of lung, liver, kidney, and heart were normal.

From these experiments it is apparent that chickens become remarkably resistant to pneumococcal infection in the first 4 or 5 days after hatching.

The Appearance of Protective Activity in the Serum of Baby Chicks

The resistance of chickens to pneumococcal infection has been attributed, at least in part, to a serum factor capable of protecting other

animal species. It was of interest to know if this protective activity is absent in the serum of freshly hatched chicks, susceptible to pneumococcal infection, and if so, whether its appearance paralleled the appearance of resistance.



TEXT-FIGURE 1. The chick's resistance to pneumococcal infection appears rapidly in the first 3 or 4 days of life.

o——o "wet" chicks. ●——● "dry" chicks (see text).

First number in brackets indicates the number of experiments; the second number in brackets indicates the total number of chicks used in that age group.

In 3 similar experiments, 50 mice were divided into 5 groups of 10 mice. In each experiment, one group of 10 mice received an intraperitoneal injection of 2 ml. of serum pooled from a single hatch of "dry" baby chicks, another group received 2 ml. of 4-day chick serum, and another group, 2 ml. of adult chicken serum. One control group received 2 ml. of normal rabbit serum and another control group received nothing. All mice in each group in each experiment were challenged 4 hours after the serum injection with 10^3 type I pneumococci. The serum for each experiment was collected from a different hatch of baby chicks. The heart blood of dead mice was cultured to rule out death from other causes.

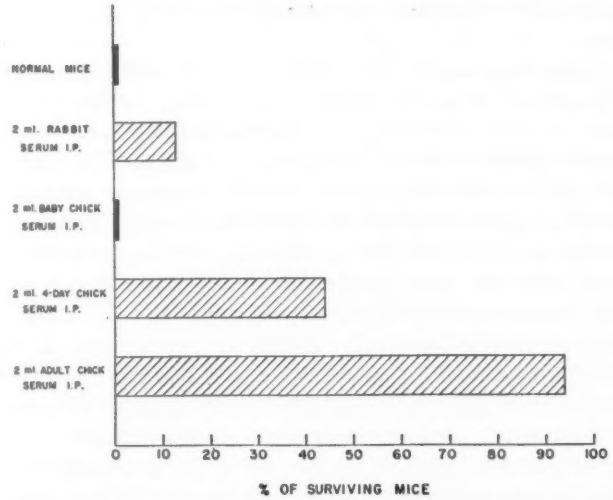
Although these experiments showed no evidence of protective activity in the serum of freshly hatched "dry" chicks, it was apparent that serum from chicks 4 or 5 days old contained protective activity sufficient to permit half of the infected mice to survive (Text-fig. 2). This is about half as effective as the serum of adult chickens, but it is significant that this level was reached in only 4 or 5 days and at the same age when

chicks were almost completely resistant. In one experiment, not included in Text-figure 2, 8 out of 9 mice were protected by an intraperitoneal injection of 2 ml. of serum from chicks 12 weeks old. This is a small group but suggests that serum protective activity is sustained after reaching a high level in the first 4 or 5 days after hatching.

The Fate of Intracutaneous Pneumococci in Adult Chickens

Animals with acquired type-specific antibody resist pneumococcal infection by agglutination, opsonification and phagocytosis of the organisms.[4] Since it has been stated that fowl serum contains pneumococcal opsonins and agglutinins and has type specificity, it seemed reasonable that fowl might also destroy injected pneumococci by opsonification and phagocytosis.

The feathers were plucked from the backs of 6 one-year-old "crossbred" hens, weighing between 2.5 and 3.1 kg. Each bird received 0.1 ml. of culture containing 3.2×10^7 highly virulent pneumococci intra-



TEXT-FIGURE 2. Considerable protective activity appears in the serum of chicks in the first 4 days of life. Each bar represents the results of 3 experiments.

cutaneously in 2 different sites, A and B, several cm. apart and on opposite sides. Each injection site was circled with India ink. Site A was removed for biopsy from chickens 1 and 2 one-half hour after injection and from site B 1½ hours after injection; site A was examined by biopsy at 2½ hours and B at 4½ hours from chickens 3 and 4; site A at 8½ hours and site B at 24 hours from chickens 5 and 6. There was

virtually no gross reaction at the site of the injections; in fact, it would have been impossible to locate them without the India ink markings. Half of each biopsy specimen was processed for microscopic study and the other half cultured (see Material and Methods). Rectal temperatures of birds 5 and 6 were taken 8 and 24 hours after the infection, and all were normal. Microscopic examination showed the following:

One-half hour after the injection, the tissues were separated, probably as a result of the mechanical effect of the inoculum and not as a result of inflammatory edema. Only a few scattered leukocytes were present, and there was some hemorrhage. Pneumococci were abundant and all were Gram-positive. There was no agglutination or evidence of phagocytosis.

At 1½ hours there were a few more leukocytes and about the same number of bacteria. There was no detectable evidence of bacterial multiplication. However, occasional pneumococci had become Gram-negative. There was no agglutination. Except for a rare leukocyte containing an ingested pneumococcus, there was no significant phagocytosis. Separation of the tissues and hemorrhage was about the same as at one half hour.

At 2½ hours, leukocytes were more abundant while the pneumococci were, if anything, fewer. Of considerable interest, however, was the appearance of many Gram-negative pneumococci. There was still no evidence of agglutination or phagocytosis; in fact, there was less evidence of phagocytosis than is seen in intracutaneous pneumococcal infections in normal rabbits. Cultures taken of these lesions were positive.

At 4½ hours there was no real change in the lesions, except perhaps that there were still fewer pneumococci, many of which were Gram-negative. There was no evidence of phagocytosis.

At 7½ hours there were very definitely fewer bacteria; it was difficult to find any at all. The few that were found were Gram-positive and only rarely was a Gram-negative organism seen. Leukocytes were more abundant, but again there was no evidence of phagocytosis. There were two pneumococcal colonies on the plate from one lesion, while the plate from the other was sterile.

At 24 hours all that remained was a few neutrophils, some macrophages and occasional fibroblasts. There were no bacteria seen in the sections, and cultures of both lesions were sterile.

In summary, there was no morphologic evidence of agglutination or phagocytosis of pneumococci in adult chickens with intracutaneous infection. There was no evidence of significant bacterial multiplication. It was of interest that within a few hours many bacteria had become Gram-negative.

Discussion

It has been assumed for nearly 80 years that fowl are natively resistant to pneumococcal infection. This assumption has persisted in spite of evidence accumulated by the early workers that would tend to indicate an acquired rather than a spontaneous or genetic origin. For example, it was reported that fowl serum protects other animals against pneumococcal infection; that it contains opsonins and agglutinins *in vitro;* that the protective substance can be selectively adsorbed for each pneumococcal type studied; and that the protective activity is confined to the water-insoluble fraction of serum globulins.[2,3] Fowl serum, at least in these respects, appears similar to the serum of animals immunized with pneumococci. It therefore seemed possible that fowl serum might contain antibodies resulting from immunization with environmental antigens and that the resistance of the fowl to pneumococcal infection, if dependent on this serum factor, is also acquired. If this were the case, freshly hatched and young chicks should be susceptible to infection and their serum should not protect other animals against pneumococcal infection. Chicks would become resistant and their serum protective only after they had become immunologically mature and had had contact with the antigen or antigens responsible for the development of anti-pneumococcal antibody.

It was therefore a surprise to find, as a result of experiments described in this report, that chicks become resistant to infection with large numbers of pneumococci within 4 or 5 days after hatching and that at this same time their serum contains considerable protective activity when tested passively in mice. It would be most unlikely that chicks would respond to any environmental antigenic stimulation in this period of time and particularly at this age. Springer, Horton and Forbes[5] showed that anti-human blood group B agglutinins were not detected in chicks until the third or fourth week after hatching. Bailey,[6] in studying normal and immune hemagglutinins in fowl for several species, found that agglutinins for rat and rabbit red cells could not be detected in the serums of chicks until they were 16 days old, while agglutinins for guinea pig red cells did not appear until 30 days after hatching. Numerous investigators have considered the human blood group A and B agglutinins in chickens to be a prototype of spontaneously originating antibodies. However, more recent evidence, especially that of Springer and co-workers,[5] would suggest that the fowl agglutinins for human erythrocytes and perhaps the agglutinins for other species, as well, are not of genetic origin, but are acquired as a result of stimulation by environmental antigens. Eventual proof that these heterohemagglutinins

are indeed the result of stimulation by ubiquitous environmental antigens would raise serious questions about the authenticity of other "natural antibodies." This would still leave considerable difficulty in explaining the appearance of anti-pneumococcal antibodies in chicks less than 4 days old on the basis of antigenic stimulation. The possibility does exist that this protective substance is passed from the mother to the chick via the yolk sac. This explanation is likewise unsatisfactory. In those instances where it has been shown that antibody passes from the mother to the chick via the yolk sac, antibody appears in the chick's serum with the absorption of the yolk and reaches its highest level at the time of hatching and then progressively declines over the next several days.[7] In contrast, the resistance of chicks to pneumococcal infection (Text-fig. 1) and the protective activity of chick serum rises rapidly in the first few days and is then sustained at high levels for the life of the bird.

The appearance of serum protective activity paralleling the appearance of resistance to infection would suggest that the serum factor plays a significant role in the chicken's resistance to pneumococcal infection. If fowl's serum "antibody" is acquired, one might expect injected pneumococci to be destroyed by opsonification and phagocytosis. We were unable, however, to find any evidence that phagocytosis played a significant role in the destruction of intracutaneous pneumococci in adult fowl. In spite of the notorious difficulty of evaluating phagocytosis in tissue sections, there was less evidence of phagocytosis of pneumococci than we have observed in nonimmune rabbits. This was surprising and perhaps open to question, since pneumococcal opsonins have been reported in the serum of fowl *in vitro*.[2] In several experiments designed to shed light on the mechanism of mouse protection with fowl serum, we have yet to find any evidence of significant phagocytosis.[8]

If the resistance of fowl to pneumococcal infection and the protective activity of fowl serum for other species is not due to acquired antibody, then what is this protective substance and what is its mechanism of action? Bull and McKee[3] found the protective activity in the water-insoluble globulins. Using a cold ethanol fractionation procedure, we have found the protective activity mainly in the beta globulin fraction.[8] These findings would indicate that it is either a protein or associated in some way with proteins, especially the serum globulins. The mode of action of this protective substance is not clear. The experiments contained in this report, together with unpublished data, indicate that fowl and mice treated with fowl serum resist pneumococcal infection either by inhibiting multiplication or by lysis of the organisms. If pneumococci are destroyed by lysis *in vivo*, it would be curious since there is no evi-

dence that fowl serum has any lytic activity *in vitro*.[2] This is in contrast to other natural antibacterial systems such as properdin, lysozyme, lysins, or leukins which are noted for their lytic action *in vitro*.[9] It should be pointed out that the anti-pneumococcal system in fowl may well not be unique, since all available evidence points to a remarkable similarity between the anti-pneumococcal properties of fowl and swine serum.[10]

A number of years ago Rich[11] showed that the resistance of normal rabbits to type III pneumococci was the result of the rabbit's febrile response to infection and the sensitivity of type III pneumococci to these temperatures. Fowl normally have a high body temperature, 107° to 108° F., and this could indeed play an important role in the resistance of the fowl to pneumococcal infection. However, if body temperature is important in the chicken's resistance to pneumococcal infection, it still does not clarify the nature of the protective activity of fowl serum in other animals since fowl serum is not pyrogenic in mice.[8]

Summary

Chicks less than 3 hours old are uniformly susceptible to infection with relatively large numbers of virulent pneumococci. When they have become 4 or 5 days old, virtually all chicks will survive a similar infection. This resistance apparently persists for the life of the bird.

The serum of freshly hatched chicks will not protect mice against pneumococcal infection, but if mice are treated with the serum of chicks 4 or 5 days old, nearly half of them will survive a similar infection.

Study of intracutaneous pneumococcal infection in adult chickens revealed no evidence of agglutination, opsonification, or phagocytosis.

It is suggested that the resistance of fowl to pneumococcal infection and the protective activity of fowl serum is not the result of acquired antibody, but is a naturally occurring antibacterial system. The available evidence suggests that fowl resist pneumococcal infection *in vivo* either by inhibiting multiplication or by lysis of the organisms. The differences between this and other naturally occurring antibacterial systems are briefly discussed.

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IMMUNOCYTOCHEMICAL STUDIES IN SCHISTOSOMIASIS

ZILTON A. ANDRADE, M.D.*; FIORENZO PARONETTO, M.D., AND HANS POPPER, M.D.

From the Department of Pathology, The Mount Sinai Hospital, New York, N.Y.

Immunocytochemistry has recently been applied to the demonstration within liver tissue of both antigen[1,2] and gamma globulin,[3,4] the latter probably representing antibody. The nature of the antigen as well as the antibody target has not been established. It appeared intriguing, therefore, to utilize immunocytochemistry in the study of the distribution of a known antigen and of gamma globulin in the livers of mice with experimental schistosomiasis. This is a disorder in which the granulomatous nature and the presence of lymphocytes and plasma cells in the lesions suggest an immunologic component in the tissue reaction.[5]

Specific antibodies in the serums of patients and animals infected with *Schistosoma mansoni* have been demonstrated serologically.[6-9] They have been applied in different stages of parasitism; namely, in relation to infective cercariae, diecious adults, miracidia and ova. More recently a fluorescent antibody technique has been developed for the serodiagnosis of human schistosomiasis[10]; in this, isolated cercariae have been utilized. Little is known about the localization, fate and pathogenetic implications of the schistosomal antigens in the tissues of the host.

MATERIAL AND METHODS

White mice of both sexes (Webster strain), 18 in number and approximately 25 gm. in weight, were infected with *S. mansoni* (approximately 50 cercariae to each animal). The infected mice were obtained from Dr. H. Schalie, Ann Arbor, Michigan, and from Dr. A. Cheever, Laboratory of Parasitic Diseases, National Institutes of Health, Bethesda, Maryland. Four noninfected mice served as controls. The animals were sacrificed weekly from the second through the 18th week after infection. Small blocks of liver and spleen were rapidly frozen in a dry ice-isopentane mixture at $-70°$ C. and stored at $-30°$ C. Sections were cut at 5μ in a cryostat, dried in a vacuum at 5° C., fixed in dehydrated acetone for 10 minutes, washed 3 times for 5 minutes each with buffered saline solution, pH 7, and subsequently treated with a

Supported by the United States Army Medical Research and Development Command under Contract DA-49-007-MD-790, by Research Grant A-3846 Path. from the National Institute of Arthritis and Metabolic Diseases of the United States Public Health Service, and by The Block Foundation.

Presented at the Fifty-eighth Annual Meeting of the American Association of Pathologists and Bacteriologists, Chicago, Ill., April 27, 1961.

Accepted for publication, June 1, 1961.

* Foreign Research Fellow, United States Public Health Service, at the Department of Pathology, The Mount Sinai Hospital, New York, N.Y. Present address: Hospital das Clinicas, Salvador, Bahia, Brazil.

few drops of fluoresceinated serums or antiserums. They were then washed 3 times for 5 minutes, mounted in phosphate buffered glycerol and examined within 12 hours with a Zeiss fluorescence microscope, utilizing an Osram 200 light source, 2 BG-12 exciter filters and one OG 5 barrier filter. Super Anscochrome film, tungsten type, was used for photomicrography; black and white photographs were made from Anscochrome slides.

Anti-mouse gamma globulin was prepared in rabbits using as antigen gamma globulin obtained from mouse serum by precipitation with ammonium sulfate. This fraction was subsequently purified through a DEAE cellulose column.[11] The specificity of the antiserum was checked by the agar double diffusion technique.[12] The gamma globulin fraction of the antiserum was conjugated with fluorescein isothiocyanate (N.B.C.).[13] In addition, slides were treated with antiserum mixed with rhodaminated * bovine albumin [14] to decrease the nonspecific uptake of fluorescein. To avoid nonspecific fluorescence of eosinophils, the antiserum was absorbed with red bone marrow powder and sections were pretreated with normal mouse serum.

To study the localization of antigen in tissues, globulin from serums of 22 patients infected with *Schistosoma mansoni* and of 15 controls was isolated by ammonium sulfate precipitation and conjugated with fluorescein isothiocyanate. Fluoresceinated serums and antiserums were twice absorbed with acetone-extracted pork liver powder before use. In addition, for the indirect technique, cryostat sections of infected mouse liver were treated with serums from either humans or mice infected with *Schistosoma mansoni;* this was followed by fluoresceinated rabbit anti-human or anti-mouse gamma globulin. Anti-human gamma globulin antiserum was prepared in rabbits using as antigen gamma globulin (Cohn Fraction II, N.B.C.) purified through a DEAE cellulose column.[11]

To elute the antibodies from the antigen-antibody complexes,[15] unfixed cryostat sections were treated for 2 hours with pH 3.2 citrate-buffered saline, and for control with pH 7 phosphate buffer. Sections were subsequently treated with fluoresceinated rabbit anti-mouse gamma globulin. In a few instances sections were subsequently washed thoroughly and treated with fluoresceinated gamma globulin from patients with schistosomiasis to demonstrate schistosoma antigen.

Duplicate cryostat sections were also stained with hematoxylin and eosin. In addition, paraffin sections of liver and spleen were stained with hematoxylin and eosin, Mallory's aniline blue, the periodic acid-Schiff (PAS) procedure after digestion with diastase, and silver impregnation according to Gomori.

Results

The liver of mice exhibited granulomas with or without schistosoma ova. The latter showed various stages of degeneration and a shell with bright brown-yellow autofluorescence. The material in the schistosoma ova gave a dense PAS reaction. In later stages of infection a crescent-shaped necrotic area which also gave a bright PAS reaction (Fig. 1) appeared within the granuloma. In sections treated with fluoresceinated gamma globulin from patients infected with *Schistosoma mansoni* (Table I), structures within the schistosoma ova (Fig. 2) (mature or immature, preserved or disintegrating miracidia) gave a strong apple-green fluorescence. This was also evident in adult worms (Fig. 3) within intrahepatic portal vein branches and in necrotic areas around the ova.

* Lissamine rhodamine B was donated by A. Hoffman and Co., Providence, R.I.

Sometimes a multilobulated mass in an ovum, suggesting penetration glands of the miracidium, was the only structure having strong fluorescence. Specific fluorescence also appeared in the cytoplasm of many macrophages (Fig. 4) and occasionally was noted extracellularly in the granulomas. Specific fluorescence was not observed in the spleen of infected mice treated with fluoresceinated serums from patients with schistosomiasis. Infected livers treated with fluoresceinated gamma

TABLE I

IMMUNOCYTOCHEMICAL OBSERVATIONS IN MICE INFECTED WITH *Schistosoma mansoni*

	Weeks after infection			
	0	2–4–6	8–10–12	14–16–18
	Number of animals			
	4	6	6	6
Binding with rabbit anti-mouse gamma globulin				
Liver				
Necrotic zone	−	−	±	+
Basophilic cells in granuloma	−	±	+	−
Basophilic cells in sinusoids	−	±	+	+
Spleen				
Basophilic cells in pulp	±	+	+	+
Binding with gamma globulin of patients with schistosomiasis				
Schistosomal granuloma				
Worm	−	+	+	+
Ovum	−	+	+	+
Macrophages	−	±	+	+
Extracellular	−	±	+	+

globulin from serums of hospital patients without schistosomiasis showed no binding (Fig. 5). This was also the case when the indirect technique was utilized.

Cells containing gamma globulin were encountered in 3 locations in infected mice: (a) in hepatic schistosomal granulomas; (b) in littoral cells of hepatic sinusoids; and (c) in the red pulp of the spleen. In earlier stages of infection, cells with specific fluorescence were noted at the periphery of occasional granulomas (Fig. 6). After 8 weeks few fluorescent cells lay within the granulomas. Later, when the granulomas became partially or wholly fibrotic, fluorescent cells were absent but the connective tissue showed diffuse fluorescence, much less intense in control sections. In the hepatic sinusoids (Fig. 7) and in the red pulp of the spleen (Fig. 8) cells containing gamma globulin increased in number progressively with duration of the infection. This coincided with a progressive increase in weight and consistency of the spleen and with reticuloendothelial hyperplasia, plasmocytosis, chronic passive conges-

tion, lymphoid atrophy and increase in collagen and reticulin fibers. Yellow autofluorescence in the spleen, caused by lipofuscin pigment, was increased in later stages.

In conventional sections the gamma globulin-containing cells lining the hepatic sinusoids in the position of Kupffer cells and in the red pulp of the spleen had round or oval configuration, abundant basophilic cytoplasm and round, occasionally eccentric nuclei with fine chromatin and nucleoli. In addition, the spleen contained many mature plasma cells. However, not all cells with the appearance described in conventional sections contained gamma globulin.

The necrotic portions of granulomas exhibited a specific fluorescence when treated with fluorescein-labeled anti-mouse gamma globulin. This was almost abolished when the sections were exposed to a citrate buffer at pH 3.2. Specific fluorescence could be restored when the sections were re-treated with fluoresceinated gamma globulin from patients with schistosomiasis (Fig. 9).

Discussion

Serums and gamma globulin from patients with schistosomiasis showed binding to structures within the adult schistosoma and its ova as well as to material in granulomas outside the ova. This observation indicates that recent serodiagnostic findings based on the use of isolated cercariae[10] also apply if liver tissue is used. The reaction with liver tissue may be an aid in the serodiagnosis of schistosomiasis if unknown serum is tested with tissue infected with *Schistosoma mansoni*. The relation of the state of the disease to the serum reactivity requires further investigation. The same reaction can be applied in the histologic diagnosis of schistosomiasis since with known serums, antigenic products of schistosoma may be demonstrated, either extracellularly or within macrophages, in the absence of recognizable ova in the granulomas. The method may be useful in determining the etiology of nonspecific granulomas in liver biopsy specimens from patients suspected of infection with *Schistosoma mansoni*.

The extracellular substance in the necrotic portions of granulomas seemed to be bound to antibodies, since upon treatment with acid pH its reactivity was abolished, probably by splitting the antigen-antibody complexes. A strong fluorescence reappeared when the sections were treated with fluoresceinated anti-schistosoma antibodies, indicating that the PAS-positive diastase-resistant antigen remained in place. These observations and the prevention of such necrosis by ACTH treatment[16] provide support for the immunologic nature of the tissue reaction in schistosomiasis. The antigenic character of the PAS-positive substance around schistosoma ova has been suggested previously,[5] and in other

liver disorders PAS-positive antigenic material has been demonstrated in bile.[1] The necrosis about the ova is apparently a response to antigen-antibody complexes rather than to free antigen; the solubility experiment suggests that an antibody is bound to an antigen in this location. Others have demonstrated the tissue damaging effects of antigen-antibody complexes.[17]

Granulomas frequently represent immune responses in tissue, and hepatic granulomas occur in a variety of conditions conventionally considered to be hyperergic in nature.[18] The demonstration of antigen in the reactive site supports this concept. Moreover, the detection in the cells of schistosoma granulomas of gamma globulin similar to that seen about granulomas induced in other conditions[19] also points to an immunologic process. The presence of gamma globulin similar to that in granulomas in basophilic Kupffer cells may also indicate hypersensitivity. These cells seem to show transformation to plasma cells despite their littoral position. This has also been observed in examples of human cirrhosis.[3] The basophilic cytoplasm in cells containing gamma globulin in the liver and spleen suggest local formation rather than phagocytosis of protein as indicated previously.[3]

Splenic enlargement appears in mice with schistosomiasis very early in the course of the infection. This seems to be not only the result of portal hypertension but also of reticuloendothelial hyperplasia, presumably on an immunologic basis in view of the many gamma globulin-containing cells in the red pulp. Occasionally, in an otherwise typical "pipestem fibrosis" of hepatic schistosomiasis,[20] fine but long connective tissue septums are noted; these resemble the pattern seen in inactive postnecrotic cirrhosis.[21] Recently studied cases of human schistosomiasis have shown accumulations of plasma cells and lymphocytes, presently designated as "immunological competent cells."[22] These are similar to the collections seen in postnecrotic cirrhosis or chronic active hepatitis[23] and might conceivably indicate an immunologic basis for the self-perpetuating hepatic process in schistosomiasis. Self perpetuation in typical postnecrotic cirrhosis, particularly that of unknown etiology, has been related hypothetically to an immunologic process.[24–26] Demonstration of an established antigen and of gamma globulin formation in schistosomiasis might represent a useful model for studies of other types of cirrhosis in which the mechanism of self perpetuation has not yet been established.

Summary

The gamma globulin in mesenchymal cells in the liver and spleen and hepatic granulomas of mice infected with *Schistosoma mansoni* may be seen by immunocytochemical methods to increase in proportion to the

development of the granulomas. Serums from patients with schistosomiasis were observed to bind antigenic substances in adult worms and ova as well as material derived from them.

The usefulness of a serodiagnostic method based on this principle and its value in the histologic distinction of granulomas is apparent. The antigen in necrotic portions of the granulomas appeared to be bound to antibodies. The visualization of gamma globulin and antigen throws light on the pathogenesis of the hepatic lesions in schistosomiasis, exemplifies the role of antigen-antibody complexes in granuloma formation and provides a model for immunocytochemical studies of other hepatic disorders.

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[*Illustrations follow*]

Legends for Figures

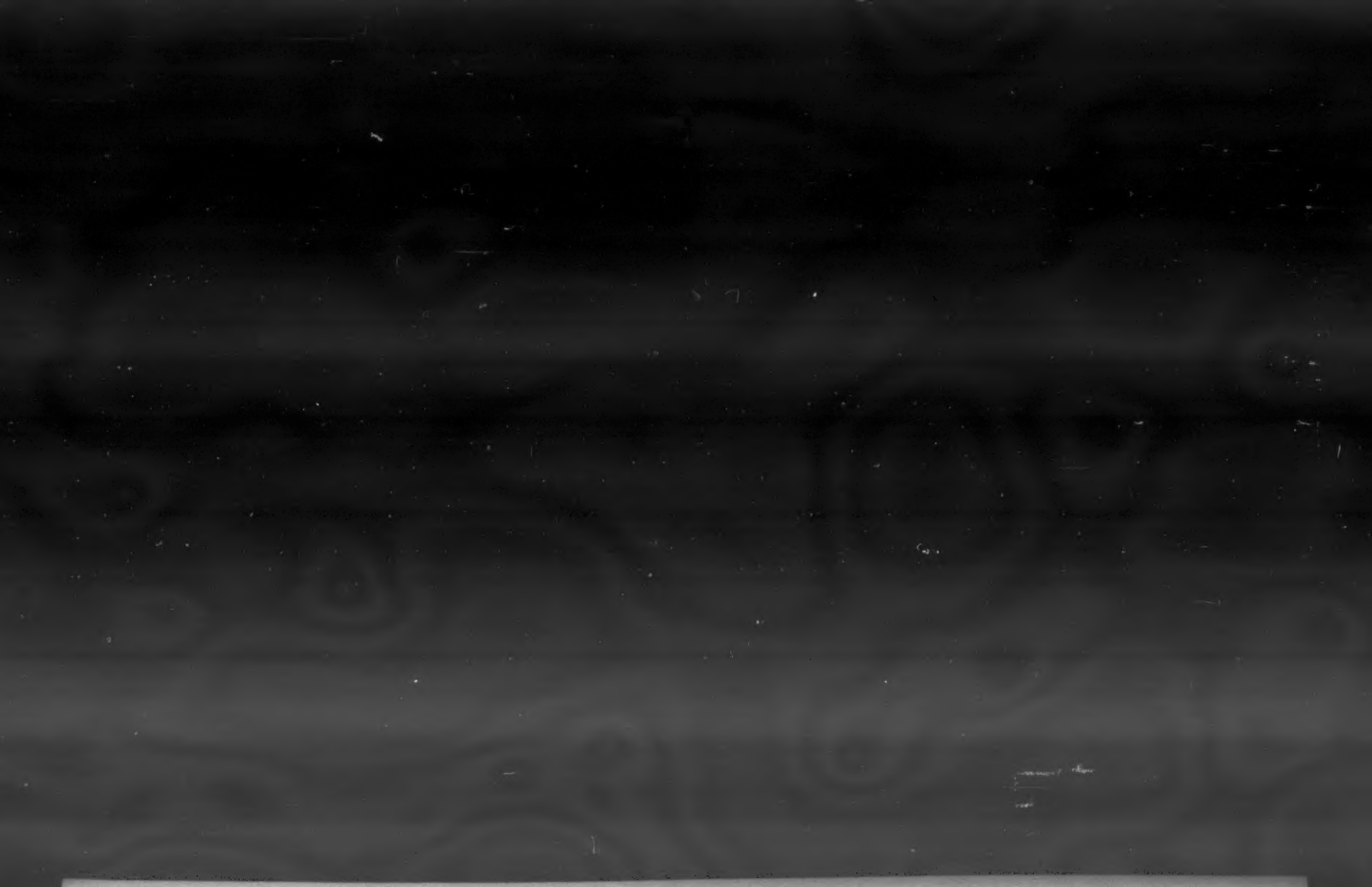
Fig. 1. Hepatic schistosomal granuloma. Note a crescent-shaped PAS-positive necrotic zone about an ovum. Periodic acid-Schiff stain. × 300.

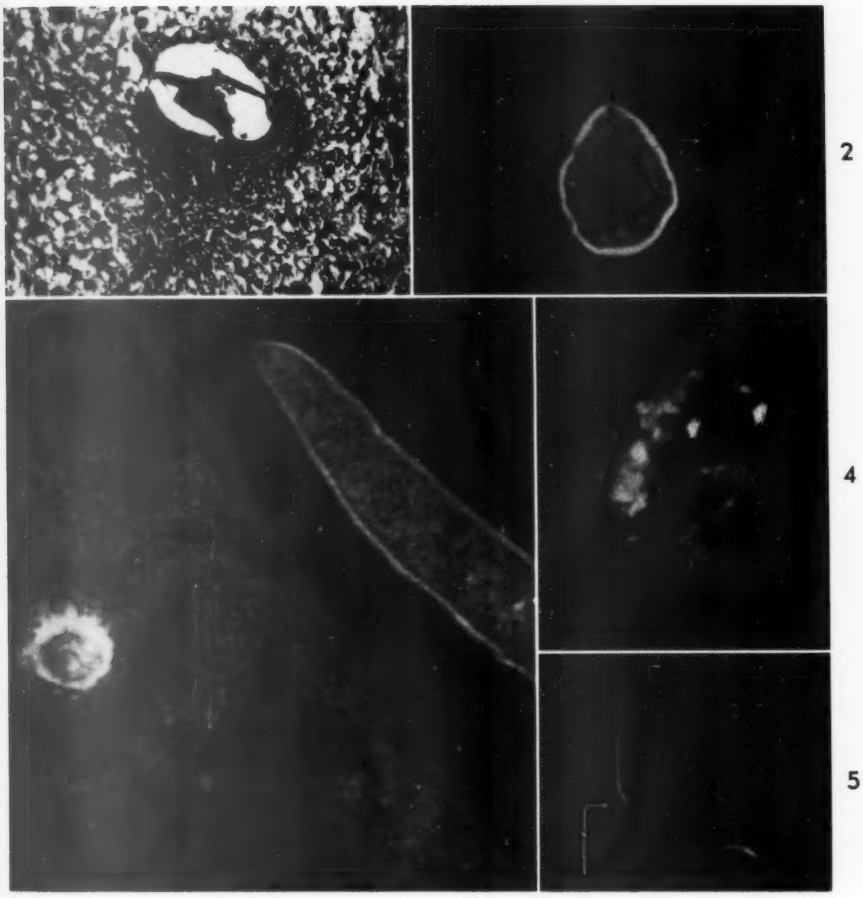
Fig. 2. An ovum in the liver of a mouse infected with *Schistosoma mansoni*. Specific fluorescence is manifest when the section is treated with fluorescein-labeled globulin from a patient with schistosomiasis. × 400.

Fig. 3. Liver of a mouse infected with *S. mansoni*. The section has been treated with fluorescein-labeled globulin from a patient with schistosomiasis. The adult worm, the ovum and a necrotic zone about the ovum show specific fluorescence. × 120.

Fig. 4. Liver of a mouse infected with *S. mansoni*. The section has been treated with fluorescein-labeled globulin from a patient with schistosomiasis. The ovum is not present in the granuloma. Specific fluorescence is seen extracellularly and in macrophages. × 400.

Fig. 5. Ovum in the liver of a mouse with schistosomiasis. There is no specific fluorescence when the section is treated with fluoresceinated globulin from a patient without schistosomiasis. The shell of the ovum shows brown-yellow autofluorescence. × 400.

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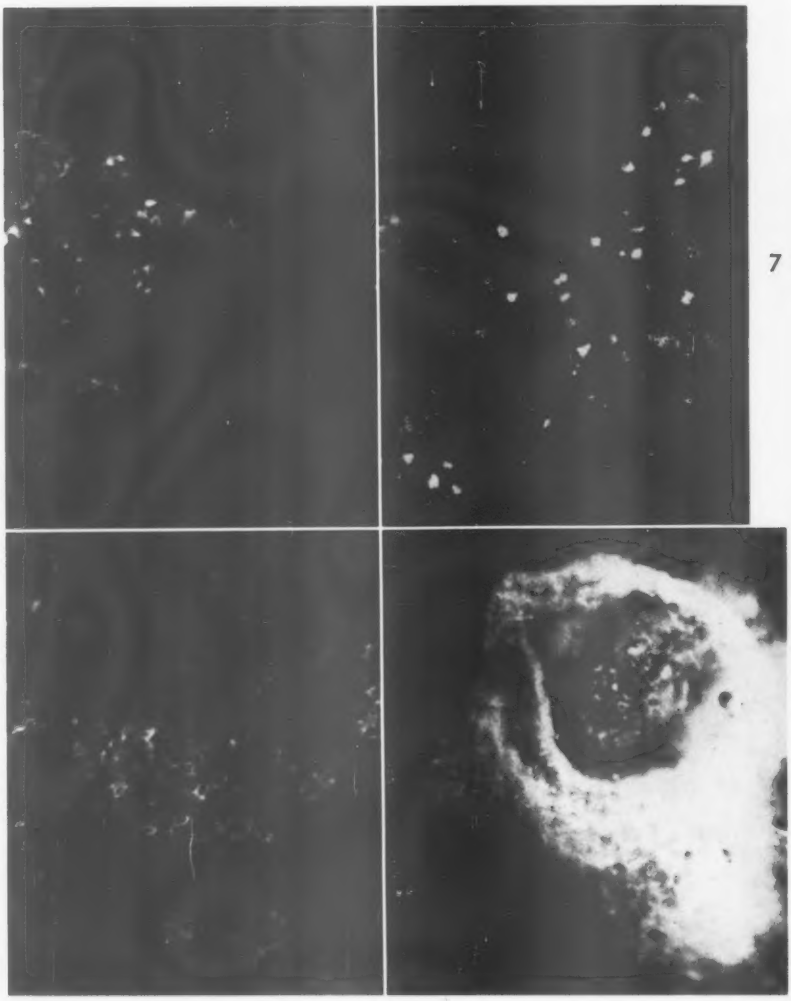
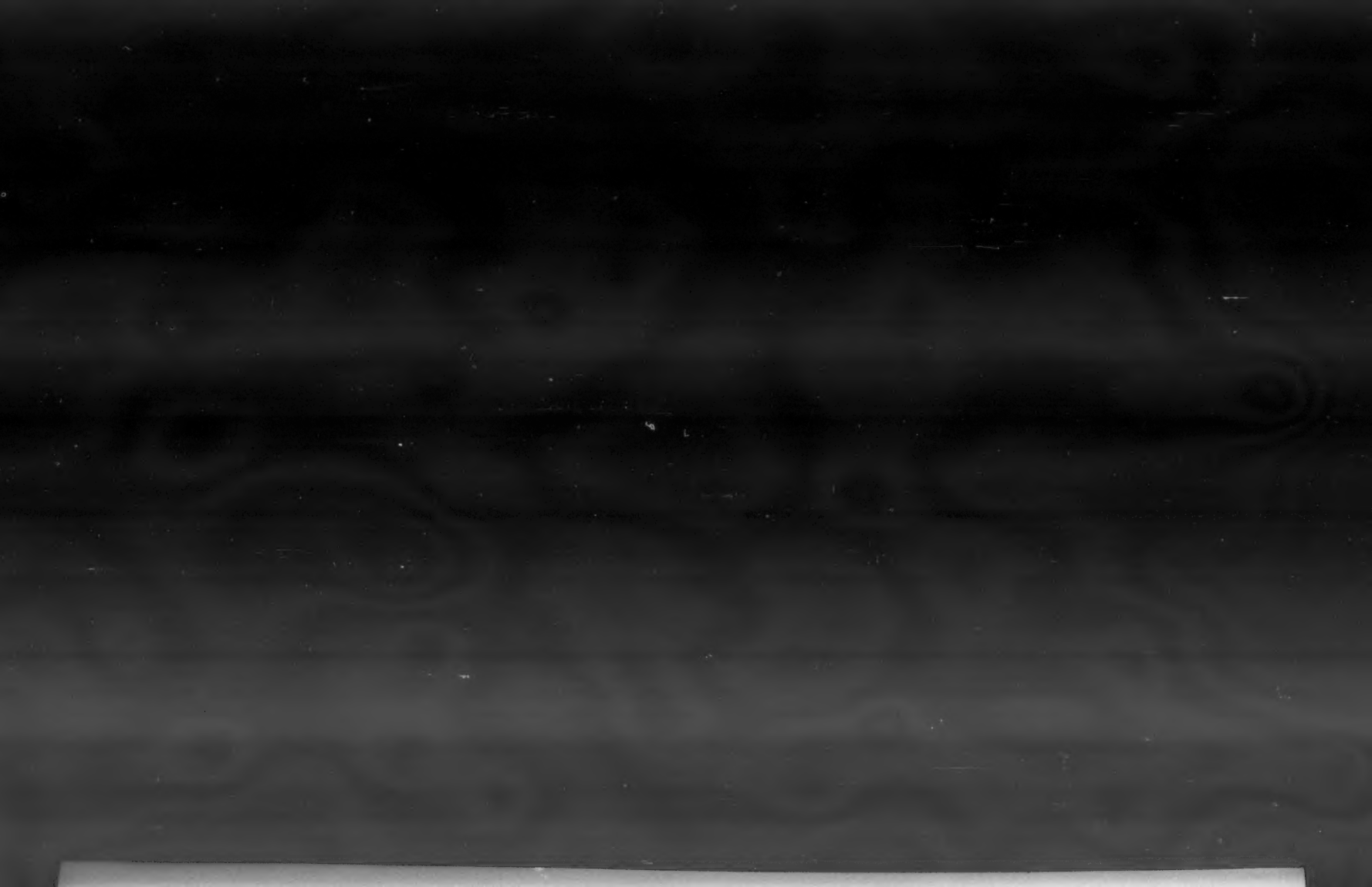


FIG. 6. A schistosomal granuloma in a mouse with infection of 8 weeks' standing. The section has been treated with fluorescein-labeled anti-mouse gamma globulin. A few gamma globulin-containing cells appear within the granuloma and at its periphery. × 400.

FIG. 7. A section from the liver shown in Figure 1. Many littoral cells contain gamma globulin in proximity to a schistosomal granuloma. × 250.

FIG. 8. Spleen of a mouse 8 weeks after infection with *S. mansoni*. Section has been treated with fluoresceinated anti-mouse gamma globulin. Many gamma globulin-containing cells appear in the pulp. × 250.

FIG. 9. Liver of a mouse 16 weeks after infection with *S. mansoni*. The section has been treated with citrate buffer (pH 3.2) followed first by fluoresceinated anti-mouse gamma globulin and then by fluoresceinated serum gamma globulin from patients with schistosomiasis. Only the latter stained the necrotic area around the ovum. × 400.



THE ETIOLOGY OF DISCRETE SPLENIC AND HEPATIC CALCIFICATIONS IN AN ENDEMIC AREA OF HISTOPLASMOSIS

MASAHIKO OKUDAIRA, M.D.; MANUEL STRAUB, M.D., AND JAN SCHWARZ, M.D.

From the Clinical Laboratories, Jewish Hospital, and the Laboratory of Mycology (Departments of Dermatology and Pathology), College of Medicine, University of Cincinnati, Cincinnati, Ohio

Calcifications of the spleen have been occasionally recorded during roentgenologic examination or at necropsy. An excellent review was published by Gray.[1] As with other early investigators,[2] he believed tuberculosis to be the most common cause of such lesions. In 1945, Christie and Peterson[3] and Palmer[4] reported convincing evidence of a relationship between the existence of nontuberculous intrathoracic calcifications and histoplasmin sensitivity. One year later, High[5] suggested that the splenic calcifications found in the course of routine roentgen examinations of the chest might represent sequelae of histoplasmosis. The same author also noted intrahepatic calcification in 3 instances.

In 1955, we reported that calcific lesions in the spleen were found in larger numbers and with much greater frequency in an endemic area of histoplasmosis than in other geographic locations. With the Gridley stain, organisms morphologically consistent with *Histoplasma capsulatum* were demonstrated in 19 of 40 examples of "typical" intrasplenic lesions. The latter were characterized by rounded configuration, a laminated structure, and were present in numbers exceeding 5 per affected spleen. The feature of lamination was somewhat variable.[6] A comparison of about 100 cases each from New York, Rotterdam, and Cincinnati revealed "typical" splenic lesions in 2.7 per cent, 2 per cent, and 44 per cent, respectively.[6] This was the first demonstration of a morphologic indication of etiology. Subsequently a relationship between skin sensitivity to histoplasmin and the roentgenographic appearance of splenic calcifications was established.[7] Subsequent studies[8–15] have provided abundant evidence that multiple concentric splenic calcifications may be considered highly suggestive of past infection with Histoplàsma.

In 1957, Young, Bills and Ulrich[16] reported that in 46 necropsies, neither acid-fast organisms nor fungi were demonstrable in smears or cultures taken from small splenic lesions or calcifications. In only a single case were organisms resembling *H. capsulatum* found in micro-

This project has been supported in part by grant E-576 of the National Institutes of Health, United States Public Health Service.

Accepted for publication, May 31, 1961.

scopic sections. More recently, however, utilizing the Grocott silver stain, Young[17] has found Histoplasma-like organisms in 10 per cent of the lesions encountered.

The present study affords a better means of evaluation since primary pulmonary complexes were examined and shown to be of histoplasmic origin. This was the case in each instance in which splenic and hepatic calcifications were encountered. Thus the potential diagnostic value of the demonstration of such foci in an endemic area of histoplasmosis becomes evident.

Material and Methods

Ninety-two consecutive necropsy examinations were performed in adults during the summer and fall of 1959 at the Jewish Hospital, Cincinnati. The patients died of a variety of disorders; in no instance was the cause of death tuberculosis, histoplasmosis or another granulomatous disease. The spleens, lungs, intrathoracic lymph nodes, and, in a few cases, the livers also were examined roentgenographically after removal from the body. By this means, in 43 instances discrete splenic calcifications were demonstrated. In 11 of these, discrete calcific foci were encountered in the liver as well.

Because of incomplete preservation of tissue, 13 of the 43 cases were excluded from this study. All primary pulmonary complexes found in the remaining 30 cases and almost all extrathoracic lesions were examined microscopically, when necessary after decalcification with 5 per cent nitric acid. Paraffin sections were stained with hematoxylin and eosin, and by the van Gieson, Kinyoun,[18] Gridley,[19] and Grocott[20] procedures. It was often necessary to recut sections and to stain them by the Grocott procedure before fungi could be found.

Results

Incidence of Splenic and Hepatic Calcifications

Among the 92 cases, there were 60 with intrathoracic calcific lesions (65.3 per cent). All of these were identified as histoplasmosis by microscopic demonstration of the organism in the individual foci. In these 60 cases, there were 43 (71.7 per cent) with splenic and 11 (18.3 per cent) with hepatic (calcific) lesions. In no instance were there hepatic lesions unaccompanied by splenic foci. Intrathoracic lesions were manifest in all.

Table I

THE INCIDENCE OF VISCERAL LESIONS OF HISTOPLASMOSIS AT NECROPSY

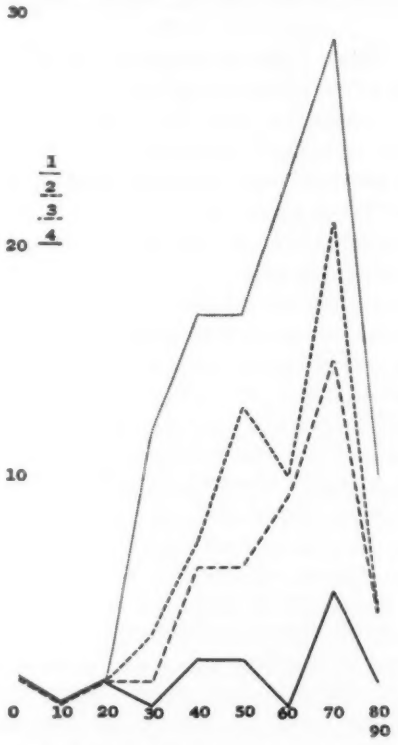
Lesion	White		Colored		Total
	Male	Female	Male	Female	
Pulmonary	30/42* (71.4%)	23/37 (62.2%)	3/6 (50%)	4/7 (57.2%)	60/92 (65.3%)
Splenic	21/42 (50%)	18/37 (48.6%)	1/6 (16.7%)	3/7 (42.9%)	43/92 (46.8%)
Hepatic	5/42 (11.9%)	5/37 (13.5%)	0/6	1/7 (14.3%)	11/92 (12%)

* Numerator, cases with calcified histoplasmic lesions; denominator, total number of cases examined.

The incidence of these processes in relation to race and sex is shown in Table I. The age distribution closely followed the over-all pattern of the pulmonary lesions and the necropsy population (Text-fig. 1).

Gross Observations

Most of the splenic lesions were spherical, 1 to 3 mm. in diameter, and gray to grayish yellow in color. Frequently a distinct lamination was noted with a solid nucleus surrounded by concentric, calcified deposits. The lesions were scattered throughout the parenchyma and on occasion lay in the splenic capsule (Fig. 1). Although the size of the lesions was usually uniform in a single spleen, occasionally there was wide variation, with both large (5 to 10 mm.; maximum 35 mm.) and small lesions in the same organ. In most instances there was dense mineralization and the lesions were hard to cut without decalcification.



TEXT-FIGURE 1. Age distribution of histoplasmic lesions in necropsy material (autumn, 1959). 1. Total number of necropsies. 2. Cases with pulmonary histoplasmic lesions. 3. Cases with splenic histoplasmic calcifications. 4. Cases with hepatic histoplasmic lesions. Age groups are arranged from 0 to 9, 10 to 19 years, etc.

In 5 cases, calcification was not a feature and the lesions appeared as centrally deposited, grayish yellow, caseous material surrounded by a fibrous capsule (Fig. 3).

The hepatic lesions (Fig. 2) were more frequently distributed in the capsule or subcapsular regions than in the parenchyma. The macroscopic and roentgenographic appearances were essentially similar to those of the splenic foci.

When the splenic lesions were conspicuously calcified, this was also the case in the pulmonary foci, the regional lymph nodes and the hepatic lesions. When splenic lesions were not calcified, mineralization was also absent from the other locations.

Histologic Observations

The histologic features of the splenic and hepatic lesions were essentially as described in our previous report.[6] Several types were recognized:

The "Early" Lesion. This was characterized by a loose, fibrous capsule surrounding a central zone of acidophilic coagulation necrosis without significant calcification (Fig. 6). Fibroblasts, small numbers of lymphocytes, and occasional epithelioid cell granulomas accompanied by foreign body giant cells were enclosed within a fibrous capsule (Fig. 7). Even at this stage, many collagen fibers appeared in the central necrotic zone extending from the fibrous capsule.

The "Old" Lesion (3 types).

1. In this form a fibrous capsule consisting of dense hyaline tissue of varying thickness surrounded a central necrotic zone (Figs. 4, 5 and 8). Calcification was generally marked in the inner half of the capsule and bone formation was noted occasionally. The central necrotic area exhibited basophilic staining and was often concentrically banded. Cholesterol clefts were frequent in the capsule and in the central necrosis.

2. Here, the core was composed principally of loose connective tissue associated with foreign body granuloma formation and cholesterol clefts.

3. These were completely hyalinized nodules without central cores.

The foregoing 3 forms either represented sequential developmental stages or were the result of tangential sectioning of certain lesions. "Early" lesions were never found in the presence of "old" ones. Although in a given case or section some lesions appeared to be necrotic and others completely fibrotic, serial sectioning revealed that most of the fibrotic lesions were actually tangential sections of a capsule surrounding an active process.

Organisms resembling *H. capsulatum* were demonstrated in the splenic foci in 26 of the 30 cases. The organisms were regularly observed

in or immediately adjacent to the center of necrotic portions. They were never found in the peripheral zone of necrosis, in granulomas or in the fibrous capsule. The number of organisms was usually small. In general, the "early" lesions contained much larger numbers than the "old" ones. They appeared singly but occasionally formed small clusters (Fig. 9). The organisms were oval and measured 2 to 3 by 2 to 5 μ. Distorted, faintly stained bodies were occasionally seen among the typical organisms. There were no unusual spore forms, and acid-fast bacilli were not demonstrable.

The interrelationships among the pulmonary lesions, the organisms in the splenic lesions, and the number of splenic lesions are tabulated in Table II. Regardless of the number of primary pulmonary lesions,

TABLE II

RELATIONSHIPS BETWEEN PRIMARY PULMONARY AND SPLENIC LESIONS

Pulmonary lesion of histoplasmosis *	Organisms in splenic lesions	No. of splenic lesions: Few †	Several †	Many †	Subtotal	Total ‡
Single primary focus	+	1	4	10	15	17 (88.2%)
	−	1§	1§		2	
Multiple primary foci	+	1	3	6	10	12 (83.4%)
	−	1§	1§		2	
"Epidemic" type[19]	+			1	1	1 (100%)
	−					
Subtotal	+	2	7	17	26	
	−	2	2		4	
Total		4	9	17		30
Per cent ‡		(50%)	(77.7%)	(100%)		

* *H. capsulatum* demonstrated in each instance.
† Few, 1 to 2; several, 3 to 5; many, 6 or more.
‡ Percentage of cases with organisms demonstrated.
§ Fibrotic hyalinized lesions.

splenic lesions were invariably multiple. Actually in 26 of the 30 cases with pulmonary complexes (87 per cent) there were multiple splenic calcifications. The number of demonstrable organisms increased with the number of foci; this held true in both the spleen and liver. Sequential sections were often necessary for the conclusive demonstration of organisms (Table III). The Grocott stain was most successful in demonstrating organisms, except in a few instances in which "fibrotic" foci were

TABLE III

IDENTIFICATION OF *Histoplasma capsulatum* ON RECUTTING OF TISSUE BLOCKS

Organ	No. of cases	First observation			Second observation			Third observation		
		+	−	%	+	−	%	+	−	%
Lung	37	34	3	91.9	37	0	100.0			
Lymph node	36	31	5	86.1	32	4	88.9	35	1*	97.2
Spleen	30	22	8	73.3	24	6	80.0	26	4*	86.7
Liver	8	5	3	62.5	5	3	62.5	5	3*	62.5

+ = organisms demonstrated; − = organisms not demonstrated.
* Only fibrotic hyalinized lesions.

observed. Recutting of these cases was not helpful, presumably because the necrotic center had been passed; thus the sections were representative of capsule rather than the active process itself.

DISCUSSION

In 1939, Reichle and Work[2] found that among 452 individuals necropsied at the Cleveland City Hospital, calcified splenic nodules were found in the spleens of 73, the livers of 47, and the kidneys of only 2. The inoculation into 20 guinea pigs of material from these lesions in 14 cases yielded positive growth of acid-fast organisms in only 3 instances. They concluded, with little justification, that calcified nodules were indeed of tuberculous nature. There was no morphologic description given of the organisms observed. In 1944, Gray[1] reported splenic calcifications in 63 of 111 unselected necropsies. In 8 of the cases there was active systemic tuberculosis; no tubercle bacilli were demonstrated in splenic foci in these individuals. Crushed material from 2 cases was injected into guinea pigs; necropsy 8 weeks later revealed no tuberculosis. In spite of this, the author concluded that the miliary splenic calcifications represented tuberculosis.

In the present group, organisms morphologically consistent with *H. capsulatum* were observed in 86.7 per cent of the calcified splenic lesions. The organisms were demonstrated in all cases in which the area of central necrosis was included in the section. Unusual forms of Histoplasma (hyphae,[21-23] globular forms,[21-24] tuberculate chlamydospores,[28] large forms,[25-29] or flagellated forms[30]) were not encountered. Schulz,[31] in a statistical review of histoplasmosis, showed a high incidence of lesions in the liver, spleen, and lung. The comparatively low incidence of hepatic calcifications in the present group of cases probably reflected incomplete roentgenographic examination.

The lack of cultural growth does not necessarily exclude histoplasmosis. Several investigators[8,32,33] have attempted culture of old histo-

plasmic granulomas with uniformly negative results. The organisms in these residual foci of histoplasmosis are presumably nonviable.[21,33,34] This explains why Young and co-workers[16] and others failed to obtain growth from calcified splenic lesions.

The Grocott stain proved to be more suitable for the demonstration of *H. capsulatum* than the Gridley stain. Positive and negative errors in the recognition of organisms were more readily avoided. Some lesions contained only a small number of organisms, and careful and repeated microscopic examinations of multiple sections through the central necrotic area, with suitable staining, were often necessary in order to demonstrate spores.

Zeidberg, Dillon and Gass[35] reported the reversion of positive histoplasmin skin tests to negative reactions. This phenomenon, which we have confirmed,[7] is most prominently observed in individuals over 40 years of age. The persistence of sensitivity to histoplasmin has been explained as an expression of a continued, active, first infection or of reinfection.[35] However, many older persons living in the endemic area of histoplasmosis react negatively to histoplasmin.[7]

Multiple discrete splenic and hepatic calcifications demonstrable by roentgenogram should be diagnostic of past infection with *H. capsulatum*. This assumes potential importance in the clinical evaluation of individuals with negative reactions to histoplasmin. The present investigation clearly indicates that in an area where histoplasmosis is endemic, calcified hepatic and splenic lesions may be presumed to indicate histoplasmosis. This presumption may be considered even more strongly if more than 5 calcified foci are observed in a given spleen and if the center of the lesions has been or is the seat of necrosis.

An explanation of the pathogenesis of these lesions is not easy and not wholly clear. There can be little doubt that the *H. capsulatum* is distributed to the spleen and liver by way of the blood stream, most likely during transitory fungemia. At this point, however, conclusive knowledge ends. One may speculate that the hematogenous dissemination results in a "filtering" of individual organisms, some of which are destroyed and others proliferate, resulting in central necrosis and permanent stigmas in the form of calcified foci. It is equally possible that the splenic lesions may result from particulate embolism with intrasplenic vascular trapping of small conglomerates of organisms with or without accompanying thrombosis.

There is evidence that transitory asymptomatic fungemia may occur. We have observed positive blood cultures of *H. capsulatum* in children without symptoms of septicemia; these patients have recovered with or without therapy. Similar experiences have been reported by others.[36]

The histoplasmic basis for these splenic (and hepatic) calcifications seems evident. It is disquieting, however, that large and numerous lesions of this nature may occur without clinical manifestations. On the other hand, splenic infarcts, even of large size, may also be asymptomatic.

Summary

Splenic and hepatic calcifications are often observed in an endemic area of histoplasmosis. Among 92 consecutive necropsy examinations in adults in the Cincinnati area, there were 43 cases with discrete, often multiple splenic calcifications, and 11 with hepatic calcifications.

The splenic calcifications in 30 cases and similar hepatic lesions in 8 were examined microscopically. In all of the lesions with active or healed central necrosis, organisms resembling *H. capsulatum* were demonstrated morphologically by special stains. The presence of multiple discrete splenic and hepatic calcifications appears to be a characteristic sequel of histoplasmosis and presumably develops asymptomatically during the period of primary infection.

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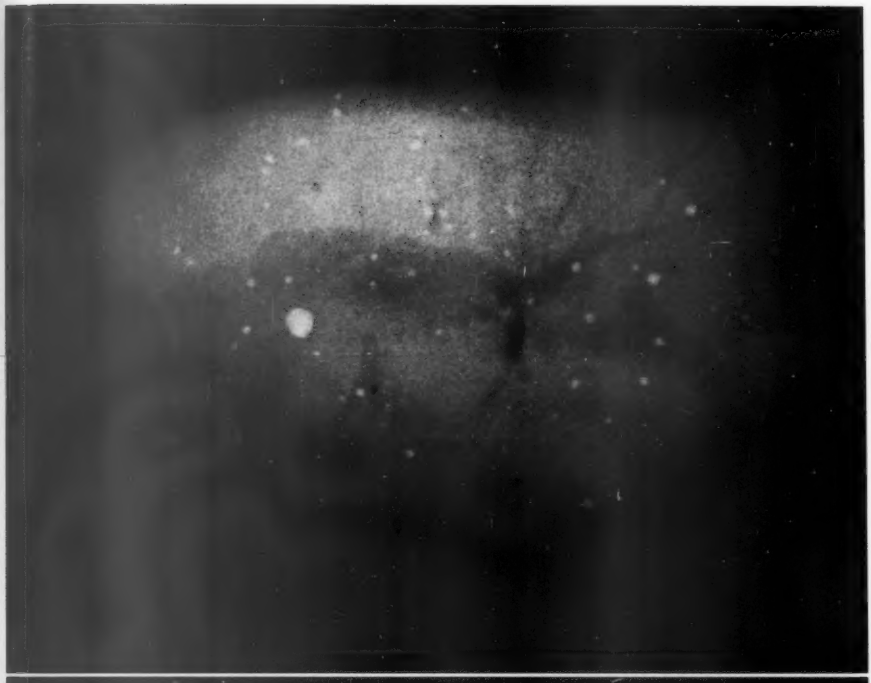
[*Illustrations follow*]

LEGENDS FOR FIGURES

Except where indicated, photomicrographs were prepared from sections stained with hematoxylin and eosin.

FIG. 1. Roentgenogram of the spleen removed at necropsy from a 41-year-old white man. Many calcifications may be seen; the largest one has "typical" concentric rings.

FIG. 2. Roentgenogram of the liver removed at necropsy from a 51-year-old white man. Numerous calcifications are visible.



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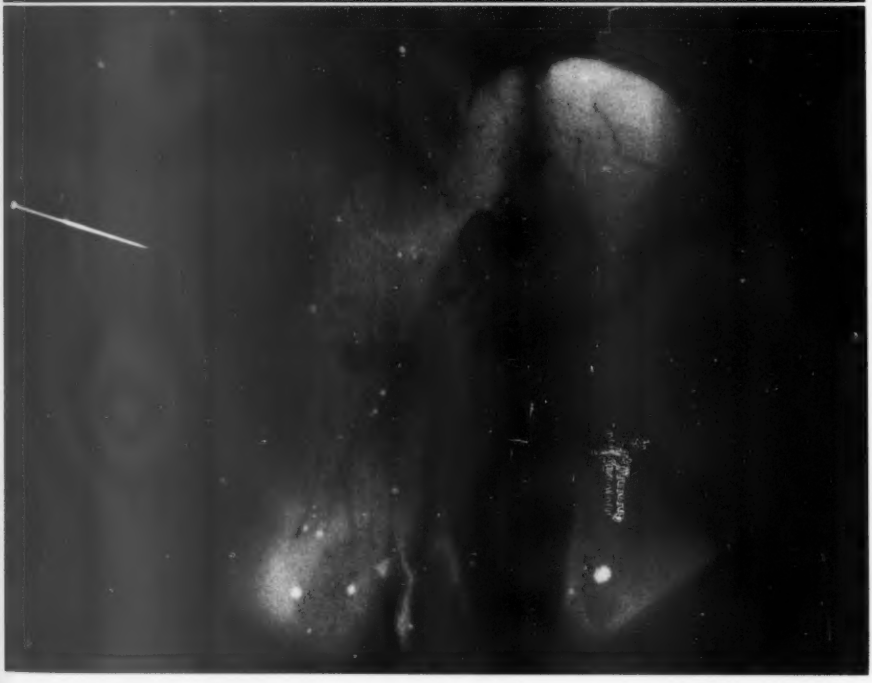

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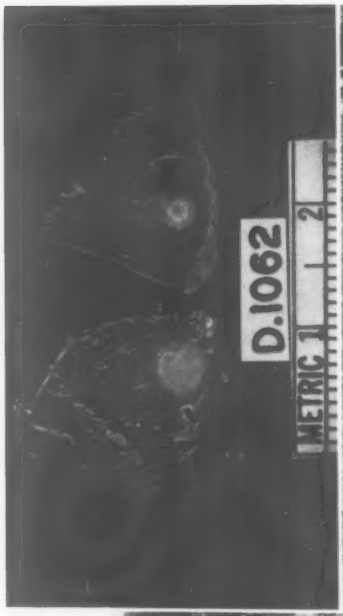

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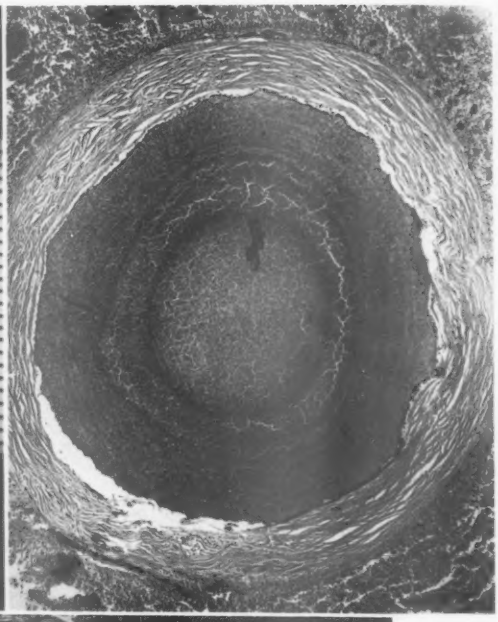

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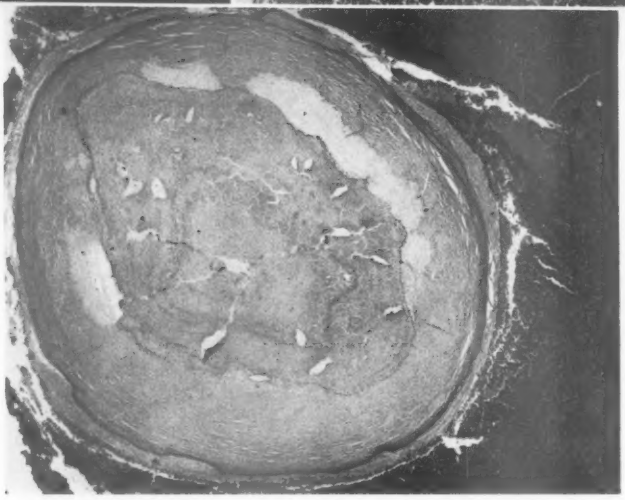

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FIG. 3. Subacute active histoplasmic splenic lesions, 59-year-old white woman. Two foci of caseous necrosis are surrounded by a fibrous capsule.

FIG. 4. Advanced splenic lesion, 35-year-old white man. A fibrous capsule surrounds the calcified central necrosis in which concentric bands of mineral deposits are visible. × 35.

FIG. 5. Old splenic lesion, 48-year-old white man, showing a hyalinized fibrous capsule. The focus is heavily calcified. Note the similarity of calcific bands to Liesegang rings, occurring in gels. × 30.

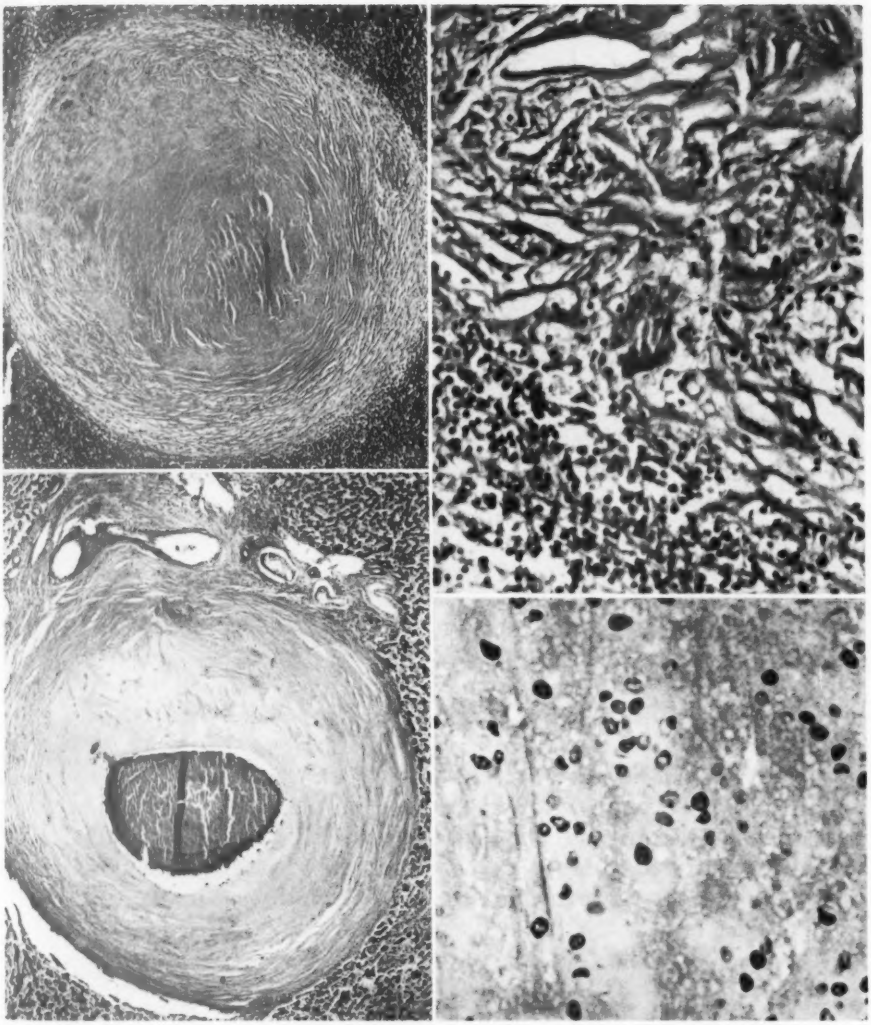

FIG. 6. A still active nodule in the spleen, 76-year-old white man. Noncalcified central coagulation necrosis is surrounded by a loose fibrous capsule. × 50.

FIG. 7. Early splenic lesion, same case as shown in Figure 3. A giant cell and epithelioid cells are demonstrated in the fibrous capsule. Central necrosis may be seen in the right upper corner. × 200.

FIG. 8. Old hepatic lesion from the same case shown in Figure 2. The centrally necrotic and calcified focus is surrounded by a thick, hyalinized fibrous capsule. × 35.

FIG. 9. Splenic focus, same case as shown in Figure 7. Numerous yeast cells which are morphologically representative of *H. capsulatum* are demonstrated in the center of the caseous necrosis. Grocott stain. × 600.

THE PATHOLOGY OF COCCIDIOIDES IMMITIS IN THE MACACA MULATTA

GEORGE P. BLUNDELL, M.D.*; MERIDA W. CASTLEBERRY, LT. COL., VC, USA, EDWIN P. LOWE, PH.D., AND JOHN L. CONVERSE, B.S.

From the United States Army Chemical Corps Biological Laboratories, Fort Detrick, Md.

Coccidioidomycosis was first reported as a disease of man in 1892 by Wernicke[1] and by Posadas.[2] The causative agent was classified as *Coccidioides immitis* in 1896 by Rixford and Gilchrist,[3] who succeeded in producing the disease in dogs and rabbits. Posadas[4] established the existence of the disorder in dogs and monkeys in 1900. In these early experiments the infectious inoculum was introduced into the experimental animals by subcutaneous injection.

Subsequently, Ahlfeldt[5] exposed guinea pigs to an aerosol of *C. immitis* arthrospores. Ophüls[6] directed attention to the preponderance of evidence favoring the respiratory tract as the most common portal of entry. His opinion, that the organism responsible for the disease in both man and animals commonly came from soil, was supported by Stewart and Meyer[7] when they isolated *C. immitis* from soil in 1932. The work of many investigators, including Dickson,[8,9] Powers and Starks,[10] and Forbus and Besterbreurtje[11] established the disease in man as primarily and predominantly a pulmonary disorder.

The production of pulmonary coccidioidomycosis in animals has included the use of inhaled wet[12] and dry[13] aerosols of the fungus, as well as the instillation into the nasal passages and the trachea of fluid containing the fungus. In addition to the studies with guinea pigs, rabbits, dogs, and monkeys already mentioned, Tager and Liebow[14] investigated the condition as it occurs in mice following intranasal instillation, and Biddle, Butt, Jacobson and Kessel[15] reported on the pathogenesis of it in the monkey following the intratracheal injection of wet arthrospores.

The fungus exists free in nature in the form of dry arthrospores in the soil and is capable of producing pulmonary infection in mammals following inhalation. This report is concerned, therefore, with "naturally" induced pulmonary coccidioidomycosis as it was investigated in its acute and chronic forms by exposing *Macaca mulatta* to a measured aerosol inhaled dose of the dry arthrospores of *C. immitis*. Since it had

Accepted for publication, May 17, 1961.

* Present address: Oscar B. Hunter Memorial Laboratory, Washington 6, D.C.

been observed that this species of monkey might die as early as the eleventh day following exposure to such an aerosol, necropsies were performed at varying intervals up to the eleventh day. Other animals were allowed to proceed to spontaneous death; the surviving monkeys were observed for one year and then necropsied.

Material and Methods

Macaca mulatta monkeys of both sexes and of an average weight of 3 kg. were used in each of the two experiments. They were subjected to detailed necropsy examination. Tissues were fixed in 10 per cent formalin solution and stained by the Giemsa method. The Gomori methenamine silver and the periodic acid-Schiff (PAS) stains were applied to the sections of lung as an aid in the identification of the fungus.

Coccidioides immitis, strain *Silveira,* was grown on solid medium, the aerial arthrospores were harvested as a uniparticulate, dry powder, and aerosolized in an exposure chamber by means of compressed air. Details of growth, aerosolization and calculation of theoretic inhaled doses may be found elsewhere.[10]

A total of 46 monkeys were exposed in two groups. The first experimental group served to establish the effect of the size of the dose on the severity and extent of the disease in monkeys exposed to aerosols of dry arthrospores. The second experiment was designed to determine the sequence of events in the tissues from the twelfth hour post-exposure to the eleventh day. The latter time was the date of the earliest spontaneous death among the animals in the first experimental group.

The initial experiment involved 28 monkeys. They were divided into 4 exposed groups of 5 animals each, with the remaining 8 animals as cage and room controls. Each group was exposed to an aerosol providing the following inhaled doses of arthrospores: group I, 10,000; group II, 1,000; group III, 100, and group IV, 10. An additional unexposed monkey was placed in the cage with an exposed monkey from each of the first 3 groups as a "cage control" to determine the transmissibility of the disease. Five additional monkeys were placed in cages to serve as "room controls" in the same room with the exposed animals.

The second experiment included 18 monkeys. Four monkeys were exposed to the aerosol containing a calculated dose of 5,000 dry arthrospores. This dose was chosen to increase the chances of finding the arthrospores in the lung. Two of the monkeys were sacrificed and necropsied 12 hours post exposure and 2 at 24 hours. The remaining 14 monkeys inhaled approximately 1,500 arthrospores, a dose more than adequate to assure the establishment of pulmonary coccidioidomycosis. These monkeys were sacrificed and necropsied on the third, fifth, seventh, ninth and eleventh days post challenge. Combuthal® [a combination of sodium ethyl (1-methylbutyl) thiobarbiturate and sodium ethyl (1-methylbutyl) barbiturate] was given intravenously to kill the monkeys.

In a third experiment, 5 additional monkeys were given 1,500 arthrospores intravenously. They served as a control group to provide information on the time of the development and appearance of lesions resulting from hematogenous dissemination of the organisms, as distinct from bronchogenic lesions. The monkeys were sacrificed on the fourth, fifth, sixth, seventh, and tenth days after challenge.

Results

The results of this study are presented in the sequence of events as they were observed to occur once the dried arthrospore had entered the lungs, rather than as the two separate experiments described.

Pathologic Changes Observed at Intervals During the First 11 Days after Exposure (Sacrificed Animals)

Observations 12 and 24 Hours after Exposure. Multiple sections of the lungs from the 2 animals sacrificed at 12 and the 3 animals at 24 hours after exposure showed no gross or microscopic tissue changes attributable to the presence of the fungus. Macrophages were present in the alveoli in many areas, and the bronchi contained traces of mucin and cellular debris, but all of these were within the limits of normal. A few slender barrel-shaped bodies (Figs. 1 and 2), which could qualify as arthrospores, were found in both the 12 and 24 hour groups. They resembled the arthrospores of the Silveira strain of *C. immitis* as seen in control smears stained by the PAS and methenamine silver methods, but none were accepted as unquestionably of fungal nature. Tissues from organs other than the lungs showed no alterations.

Observations 72 Hours after Exposure. No monkeys were examined on the second day after challenge, but 3 were examined on the third day. In the lungs of 2 monkeys a developing spherule was found within a terminal bronchiole. The spherules were young, for their cytoplasm was limited to a narrow zone beneath the outer covering. One spherule was surrounded by neutrophils and macrophages, and the entire mass filled an alveolus (Fig. 3). The other spherule rested on the muscle layer and appeared embedded among cells of the cuboidal epithelium in this segment of the bronchial tree (Fig. 4). The lung of the other monkey also contained spherules, but they were within alveolar spaces. These spherules were more mature than those in the bronchioles, and their cytoplasm was granular, as if it was segmented into endospores. The spherules were surrounded by macrophages with granular brown cytoplasmic pigment, by smaller monocytes, and by neutrophils. No bodies were found above the terminal bronchioles. No arthrospores were identified.

Observations 5 Days after Exposure. Again, in the 3 animals examined 5 days post challenge, there were no lesions detected on gross examination. Microscopically, however, the lungs contained numerous minute cellular lesions distributed uniformly throughout. They were composed of dense collections of neutrophils, lymphocytes, and macrophages and filled 6 to 10 alveoli, as seen in cross section (Fig. 5). In PAS-stained sections the lesions were found to contain numerous slightly oval to spherical bodies with a pink to dark red-staining periphery. The internal structure in most of the bodies was limited to a single eccentrically placed red dot which was accepted as the nucleus (Fig. 6). The methenamine silver stain was similar, with the equivalent portions being

dark gray to black. The bodies were 8 to 10 μ in diameter and were considered to be endospores just beginning to undergo swelling or growth. A few were also found in small traces of exudate within the lumen of a major bronchus.

Some of the lesions lay beneath the serosal surface, but the pleura had reacted only to the extent that a few cells of the serosal surface were enlarged. No fungal forms had penetrated the pleura.

In addition, an isolated mature spherule was present in an alveolar space in 2 of the monkeys. The cytoplasm in these was granular; they were thus mature enough to contain endospores. None of the compactly enclosed endospores, however, were quite as large as the bodies designated immature spherules in the lesions described above.

Observations 7 Days after Exposure. The animals in this group, as well as the other remaining animals, had an average temperature rise of 2.5° F. The lungs in the 3 animals contained grossly visible focal lesions, which were dark red and approximately 1 mm. in diameter. Microscopically, in some instances the lesions were twice the size of those in the 5-day post-challenge cases, and on cross section involved as many as 12 to 16 alveoli. The majority were discrete, and some compressed the alveoli about their peripheries. Neutrophils were predominant and filled intact alveolar spaces; a few lymphocytes and macrophages were also present. The lesions evidenced a very limited amount of alveolar wall necrosis and also hypertrophy and hyperchromatism of the alveolar lining cells. Many immature and some mature *C. immitis* spherules were distributed both in the lesions and in the purulent bronchial lumen exudate. Concentrations of neutrophils were common about ruptured mature spherules. The endospores at this time had strongly PAS-positive material adherent to their outer covering.

The lesions were uniformly distributed throughout the lung with most of them associated in relation to a bronchiole. The surface cells of the pleura overlying peripheral lesions were plump, hyperchromatic, and at least 4 layers thick. Exudate and numerous spherules filled intermediate-sized bronchi adjacent to lesions and also extended into the larger bronchi. Some of the peribronchial lymph nodes had dilated subcapsular sinuses.

Observations 9 Days after Exposure. On the ninth day post challenge the monkeys had a temperature of 104.5°, 105.4° and 105.0°. Two of these were necropsied, and the third was held until the eleventh day. Grossly, the lungs of both animals were mottled yellowish white to dark red, with most of the surface being made up of many grayish brown nodules which measured up to 3 mm. Numerous fibrinous adhesions were present. The lesions on the cut surface, while larger than those

observed previously, were still focal. Microscopically, the lesions in cross section were 3 to 5 times larger than the 7-day lesions. Spherules of various ages, fragments of spherules, and endospores were distributed through them. In addition to the changes described on the seventh day, many had varying amounts of amorphous, eosin-staining necrotic material in the centers as well as fragmented neutrophils and other cellular debris. The central portions of other lesions were filled with intact neutrophils, and some lesions contained small conglomerates of eosin-staining necrotic material with a coagulative texture. Multinucleated giant cells of the Langhans type were present. This, combined with the presence of fibroblasts along the course of alveolar walls and the increase in size and hyperchromatism of cells lining the surrounding alveolar spaces, gave a chronic proliferative character to the lesions. Even so, the exudate in the bronchi remained purulent.

Most of the lesions had a zone of intra-alveolar edema about them. Macrophages were numerous within the alveoli involved in this change. The pleura overlying peripheral lesions was covered with a dense layer of fibrin into which young fibroblasts grew.

One of the two monkeys showed numerous foci of caseous necrosis in the peribronchial lymph nodes and spleen. Langhans-type giant cells were numerous here. There was also caseous necrosis in a parapancreatic lymph node. The lesions in the liver were small, widely scattered, and composed of epithelioid and multinucleated giant cells. No fungi were found in the Giemsa-stained section, and only a single small spherule was found in a PAS-stained section of one hilar lymph node. These tissues were stained by the Ziehl-Neelsen technique, but no acid-fast bacilli were found. All monkeys had been skin-tested with old tuberculin prior to use in the experiment and had been found to be tuberculin-negative. The presence of extensive necrosis and of the numerous giant cells outside of the lung this early in the disease in one monkey of the group exposed by the respiratory route was exceptional.

Observations 11 Days after Exposure. The last animal to be killed and necropsied closely resembled the 9-day animals. Grossly, the lungs were similar; the pleural surface was yellow-white to dark red, with multiple, discrete, grayish brown nodules as large as 4 to 5 mm. There were multiple coarse, stringy, fibrinous adhesions. Mucoid, whitish yellow fluid filled many of the bronchi.

The microscopic appearance was also similar although the changes were more pronounced. The lesions were larger and many had coalesced. Remnants of spherules were prominent in all lesions. The central portions of empty spherules were filled with neutrophils. Edema was present, especially at the periphery of and between lesions. Numerous

bronchi were filled with a dense cellular exudate which contained many spherules. A few small multinucleated giant cells were present along the margins of the dilated subcapsular sinuses of peribronchial lymph nodes.

Lesions Observed in Animals Dying at Intervals from 11 to 30 Days after Exposure

Observations 11 Days after Exposure. The gross appearance of the lungs of the monkey that died on the eleventh post-challenge day was essentially similar to that in the monkey sacrificed on the eleventh day.

The microscopic appearance was also similar, with one possible exception. Pulmonary edema appeared to be greater in the lungs of the animal dying spontaneously, and also there was more dense, highly cellular exudate in the bronchi and the trachea. It was estimated that these cellular and necrotic lesions involved over two thirds of the lung parenchyma, while practically all of the remainder of the lung showed intra-alveolar edema. Very little of the lung contained air.

The peribronchial lymph nodes had dilated subcapsular sinuses which contained numerous macrophages. An occasional young spherule could be found in PAS-stained sections, but no focal lesions were found.

Observations 12 Days after Exposure. The changes in the lungs of the monkey that died on the twelfth day were similar to those on the preceding day, with a few exceptions. The disease process was more confluent, and though there were centrally suppurative or abscess-like areas in which some of the alveolar walls had been destroyed, the alveoli for the most part maintained their identity. A peribronchial lymph node contained a small peripherally placed necrotic focus which contained spherules.

Observations 15 Days after Exposure. The lungs of the monkey that died on the 15th day had a completely bosselated pleural surface with multiple adhesions that were more difficult to break than in previous cases.

Microscopically, the lesions were large and confluent, and those few alveolar spaces which were not involved in the disease process were free of edema. The central suppurative areas were surrounded by caseous necrosis in several regions. Multinucleated giant cells were more numerous. Cellular exudate filled the bronchial lumens.

The spleen contained a small, necrotic lesion in which there were spherules and multinucleated giant cells. The liver contained a small lesion composed of neutrophils, spherules and fibrin threads.

Observations 22 Days after Exposure. Two monkeys died on the 22nd day. The principal difference in these monkeys and the one dying on the 15th day was the extensive central necrosis in most of the lesions. The

necrotic material was eosin-staining and included ghost outlines of the alveolar walls, a few neutrophils and nuclear debris as well as intact and fragmented spherules. The periphery of the lesions and the non-necrotic diseased areas had thickened and fibrosed alveolar walls which contained lymphocytes and plasma cells. Multinucleated giant cells and intact spherules were also numerous, and cuboidal epithelial cells lined many bronchiole walls and adjacent air spaces.

Observations 30 Days after Exposure. The monkey that died on the 30th day had received a calculated inhaled dose of 7 arthrospores. Grossly, its lungs were discolored and bosselated, and numerous pleural adhesions were present. The multiple focal and confluent lesions in a few instances had sharply demarked, prominent caseous necrotic centers. The striking difference in this case and the one that died at 22 days was the great number of spherules in the lesions. Though spherules in all stages of development were present, the majority were of an intermediate size which did not contain endospores. They formed a ring about and were distributed through the necrotic centers while in other portions of the same lung they were present in a diffuse, uniform fashion within viable tissue.

The lesions in this animal were disseminated. There were small ones in the hilar lymph nodes, a lymph node in the left axilla, spleen, liver, bone marrow, adrenal gland, voluntary and cardiac muscles, and skin of the left wrist.

Pathologic Changes Observed at Intervals from 1 to 12 Months

The monkey that died on the 43rd day had severe involvement of both lungs, with very little normal air-containing tissue. Though the disease process affected lobules and almost entire lobes of the lung, the pattern was essentially that of a very large chronic mycotic abscess, for the necrotic central portion contained numerous neutrophils and the remaining portion was composed predominantly of fibrous tissue and multinucleated giant cells. Bronchial lumen exudate in some instances had undergone organization. Many of the bronchi exhibited tortuous pathways. The epithelium in some had proliferated to form flat polypoid masses (Fig. 7), while adjacent epithelium and the mucosa elsewhere consisted of a single cell layer, often with large hyperchromatic nuclei. Extrapulmonary lesions were found in the peribronchial lymph nodes and bone marrow.

In a monkey dying on the 49th day, the lesions had large central areas of caseous necrosis containing some cellular debris and spherules. The boundary between the necrotic debris and the concentrically arranged fibrous tissue at the periphery was more distinct in this monkey

than in those examined previously. Squamous metaplasia was present in a portion of a bronchial wall bordering one of the necrotic areas. Lesions were found in the spleen and liver; those in the spleen were large, while in the liver they were minute.

The monkey dead on the 54th day was similar to the one dying on the 49th day. Spherules seemed more numerous in the lung, and the bronchi contained large quantities of purulent exudate. It was at this stage that growth of fibroblasts was directed into the necrotic material and a palisading pattern extended inward from the fibrous tissue border of the chronic abscesses. Calcium was also deposited in the center of one lesion. This monkey and the one dead on the 49th day developed skin lesions with thrombi and slight hemorrhage deep in the dermis. The more superficial portions of the dermis and epidermis were not well preserved.

At 75 days lesions were found to be scattered and the intervening lung parenchyma was essentially normal. The largest lesions were necrotic, the intermediate lesions predominantly suppurative, and the smallest lesions were composed of a mixture of epithelioid cells, multinucleated giant cells and neutrophils. Lymphocytes were prominent, especially in the peripheral portions. Exudate was still present in the lumens of the bronchi.

At 123 days an additional feature was the aging of the connective tissue in pulmonary lesions so that there was a hyaline texture. The lungs in the monkey that died on the 270th day contained extensive hyaline scars. Numerous small epithelium-lined bronchial spaces within the scars afforded a lacy appearance.

At 346 days large caseous necrotic lesions were encountered; several contained cavities that involved practically entire lobes. This animal also had a large mycotic abscess which involved the anterior portion of the right cerebral hemisphere. A most interesting feature was the presence of filamentous forms in the caseous debris lining the cavities. The filaments appeared to grow from spherules (Fig. 8) and also from germinating endospores.

The 6 surviving animals were necropsied 378 days after exposure. Each animal had active pulmonary disease in the form of ulcerative coccidioidal bronchitis. The most numerous lesions were fibrous tubercles containing either multinucleated giant cells or calcium or both. In some there were abscesses but no caseous necrosis was encountered. Spherules were present; there were no hyphae.

Pathologic Changes in Monkeys Receiving Intravenous Inoculation

In each monkey that received the aerosol challenge, the multiple lesions which developed in the lungs were attributed to arthrospores

transported in the aerosol. There was no way, however, to rule out the possibility that some lesions developed as a result of hematogenous or lymphatic spread. In an effort to determine what difference, if any, existed in pulmonary lesions of aerosol-exposed and intravenously injected monkeys, 5 were given 1,500 arthrospores intravenously. They were necropsied on the fourth, fifth, sixth, seventh, and tenth day after exposure.

On the fourth day a spherule was present in the spleen with no tissue reaction associated with it. It had an internal zone of segmented cytoplasm. A minute lesion was evident in the liver, but none appeared in the lung. The animal examined on the fifth day did have pulmonary lesions that contained spherules. The largest was diffuse enough to involve approximately 40 alveoli. The inflammatory reaction in these lesions was more homogeneous than in those resulting from aerosol challenge. Focal lesions were also present in the spleen and liver.

Lesions of the same size and distribution occurred in the animals necropsied on the sixth and seventh days; the lesions in the liver and spleen were larger and more numerous. On the tenth day a few large pulmonary lesions were manifest, but in none was there extensive necrosis or multinucleated giant cells. The lesions in the spleen were large and numerous, and those in the liver were small and abundant.

None of the 5 animals had exudate in the bronchial lumens.

Discussion

This study of the pathogenesis of coccidioidomycosis was designed to follow the tissue responses in a host, the *Macaca mulatta,* during the course of the disorder, including its incubation period, the early phase of the disease proper, and the chronic course through a 12-month period.

The Parasite as It Occurs in the Mammalian Host

The fungus was introduced into the lung by an aerosol containing dry arthrospores, free of spherules and mycelial filaments. The arthrospores were never positively identified in tissue, but structures resembling them could be located in the lumen of the bronchi and in alveoli at 12 and 24 hours after exposure. No spherules were detected in the lung samples, though Tarbet, Wright and Newcomer, in 1952,[17] did find early spherule formation in mice 24 hours after the intraperitoneal injection of mycelia. By the third post-challenge day, however, spherules were found in our monkey hosts. Their development by the third day corresponded with Ophüls' observations[6] in his rabbit number 4. An immature spherule was located in the lumen of a terminal bronchiole and another was adherent to the wall of a bronchiole of similar size denuded of its cuboidal epithelium. The two spherules found in the

alveoli were more mature and were completely filled with cytoplasm. It thus appears that the individual dry arthrospore, which exists in hot, arid regions, is capable of establishing itself in the lung of the monkey and progressing to proliferation.

The restriction of spherules to the terminal bronchioles and alveoli suggests that those arthrospores which escaped the filtering action of the nose and reached the ciliated epithelium of the bronchial tree above the level of the terminal bronchioles were effectively removed by the cilia and other actions comprising the pulmonary cleansing mechanism.[18] Arthrospores reaching the terminal and respiratory bronchioles and alveolar spaces developed into spherules within the 24 to 48 hours. Tager and Liebow[14] found that even on the fifth day almost all of the lesions in the lungs of mice inoculated intranasally were associated with terminal bronchioles. The distal localization of arthrospores within the lung and the subsequent development of lesions there constitutes an unusual feature, inasmuch as it is generally accepted that the flow of air ceases at the level of the junction of the terminal and respiratory bronchioles. The gaseous exchange beyond this point occurs by diffusion. Particulate matter should not accompany the air stream beyond this point and so should not enter the alveolus. Spherules and arthrospore-like structures, however, were observed in alveolar spaces. The shape and density of the arthrospores could be a factor in their entering the alveoli.

The fungus was observed as endospores in microscopic lesions on the fifth day. Their appearance was remarkably similar to that of Histoplasma. Forms in each of the two genera are uninucleate, according to Emmons.[19] In the monkeys the spores were round to oval with a thin outer membrane and an eccentrically placed nucleus; both stained with the PAS and methenamine silver methods. A mature spherule at this time interval appeared only as an isolated structure within an alveolus.

Endospores and spherules in various stages of development were present in focal lesions on the seventh day. A single spherule with a budlike extension was found at this time. Neutrophils were always numerous about the endospores. In a few instances a PAS-positive coating was observed on the newly released endospores. It is possible that the coating possesses a leukotaxic quality or provides the surface of the endospore with an adhesive texture. This property, coupled with the abundance of purulent exudate, makes aerogenic dissemination of intrapulmonary endospores unlikely, though they are distributed along the bronchi in the exudate.

Endospores and spherules were manifest throughout all lesions after the seventh day. From this and their subsequent locations, it is reason-

able to conclude that the endospore-spherule-endospore cycle in the monkey may, in the acute stages of a primary infection, occur during a period of 3 days or less. The fragmented walls of spherules, as well as intact spherules, were accompanied by Langhans giant cells by the ninth day.

Mycelial filaments were found in pulmonary abscesses in caseous debris in a monkey that died 346 days after exposure. Although the necrotic centers of many lesions were examined, filaments appeared only in cavitations.

The Host Tissue Response to the Fungus

In the *Macaca mulatta,* structures resembling dried arthrospores were found on bronchial epithelium and in alveolar spaces but never with an accompanying inflammatory response. It must be assumed that arthrospores act merely as inert foreign matter when in contact with pulmonary tissues. There were no evidences of significant macrophage activity or of excessive secretion of mucinous or serous fluid by the bronchial glands.

Arthrospores developed into spherules by the third day in the terminal bronchioles and alveoli, and there they became coated with inflammatory cells, including neutrophils and macrophages. A distinct lesion was first found on the fifth day. Not visible grossly, microscopically its cross section involved approximately 6 alveoli which were filled with neutrophils and a few lymphocytes and macrophages. PAS and methenamine silver stains brought out the many endospores scattered among the inflammatory cells. It should be stated again that both the endospores and the lesions exhibited close resemblance to the organisms and inflammatory reaction in histoplasmosis. It is also evident that the newly released endospores exhibited a leukotaxic action.

Grossly visible lesions appeared in the lungs at 7 days. Spherules as well as endospores were distributed throughout the lesions, at the periphery of which were metaplasia of the cells lining the alveoli as well as thickening of alveolar walls. Some evidence of this was apparent to a lesser degree at 5 days. Thick exudate, composed of neutrophils and other cells and intrabronchial exudate, continued as a prominent feature throughout most of the course of the disease.

By the ninth day, small necrotic centers appeared in many lesions. The necrotic material may have served as a pyrogen, or contained one, since fever became evident clinically. Multinucleated giant cells were present and fibrinous pleural adhesions were numerous. A spherule was found in the subcapsular sinus of a peribronchial lymph node.

The lesions were large enough to coalesce by the eleventh day, and

the surrounding alveoli were the seat of edema. In an animal that died spontaneously, intra-alveolar edema and intrabronchial exudate were prominent features. Hyaline bands of coagulated edema fluid lined some of the alveoli. The peribronchial lymph nodes now contained peripherally located spherules associated with multinucleated giant cells.

At 15 days and thereafter the lungs had a bosselated appearance because of the large size of the lesions. Segments of the bronchial tree were incorporated in portions of the lesions, and the bronchial epithelium was destroyed in many areas. A significant feature was the presence of discrete necrotic lesions in the liver and spleen.

Central necrosis of lesions was more extensive at 22 days, and there was prominent interstitial thickening of the alveolar walls between lesions. This latter feature was not necessarily attributable to the coccidioidomycosis. One gathered the general impression that the parenchyma between lesions reacted in this manner during the progressive phases of the disease. The appearance of the lungs at 22 days was comparable to that reported in a 21-day-old infant dying of acute pulmonary coccidioidomycosis (miliary dissemination within the lung), reported and illustrated by Christian and associates[20]; one illustration also appears as Figure 23 in Fiese's book.[21] In this connection the question may be posed as to whether the monkeys employed in this study were susceptible in part because they were young.

At the end of one month, lung lesions contained a large amount of frankly caseous necrotic material. Spherules were very numerous, and the disease had become generalized throughout the body. After this period, evidence of healing was more prominent. However, active lesions persisted, some increased in size, bronchial exudate was always present, and the animals died of the disease. In the monkey that died on the 43rd day, there was active proliferation of bronchial epithelium. This was manifested by segments containing large cuboidal cells with large hyperchromatic nuclei, and by other regions with exceptionally tall columnar cells which occasionally attained the form of mushroom-like polyps. At 49 days the boundary between the central necrotic material and fibrous tissue periphery was sharp, with the fibrous tissue being arranged in a concentric pattern. By the 54th day the inner zone of peripheral fibroblasts was growing into the caseous material, providing a palisade-like pattern. In the animal that died on the 123rd day fibrous tissue about the lesions had assumed a hyaline texture.

The animal that died on the 346th day probably succumbed to a large mycotic brain abscess. Mycelia in the large cavitary, caseating pulmonary lesions completed the range of fungal structures, as observed in coccidioidomycosis in man.

The 6 animals that appeared to have recovered were necropsied on the 378th day after exposure. The lesions observed in them established beyond any doubt that the arthrospores administered in aerosol were 100 per cent infective. Even to the final period of observation, all monkeys exhibited purulent bronchial exudate containing spherules and endospores. All cage control animals were free of any evidence of disease due to *C. immitis*.

No tissue reaction occurred about arthrospores, but suppuration was regularly observed in the presence of endospores. It was impossible to determine whether the surface covering of spherules was wholly inert or whether it was capable of injury or the stimulation of tissue proliferation. Several different reactions were encountered throughout the course of the disease. Spherules coated with neutrophils and macrophages were observed free within alveolar or bronchial lumens, or lying against the surface of these structures. They were also found attached to the alveolar surface or lying within the terminal bronchiolar muscular coat. In other instances, dense focal collections of spherules of an intermediate size appeared in viable tissues, the seat of chronic interstitial pneumonia, with greatly thickened alveolar walls. Here, fibroplasia was a prominent feature. Spherules were also scattered both within areas of caseous necrosis and in great numbers at the margin of such lesions, where caseation and fibrous tissue were in contiguity. In still other regions, multinucleated giant cells of the Langhans rather than the foreign-body type were associated with both intact spherules and fragments of the outer spherule membranes. It seems obvious that the spherules were not inert. Rather than a necrotizing effect, however, they appeared to be irritative and productive of proliferative reaction.

Summary

1. *Macaca mulatta* exposed to an aerosol containing a carefully determined dose of individual, dry *Coccicioides immitis* arthrospores were found to respond as follows during the first 15 days after exposure: The lungs contained spherules in the terminal bronchioles and alveoli by the third day. Lesions of microscopic size, filled with endospores and bearing a remarkable resemblance to the organisms and lesions of histoplasmosis, were present by the fifth day. A clinical response, characterized by fever, began on the seventh day. The lung lesions at this time were grossly visible as 0.1 cm. dark red nodules and had necrotic centers. Lesions appeared in organs other than the lung by the 15th day.

2. During the initial 30-day period the lesions progressed from an acute focal pulmonary process to one exhibiting disseminated lesions with both acute and chronic features.

3. The tissue responses observed were similar to those in other chronic infectious pulmonary diseases; the presence of the various forms of *C. immitis* served as distinguishing features.

4. Lesions developed in the spleen and liver at an earlier time in animals inoculated intravenously than in those exposed to an aerosol inoculum.

5. The disorder was found to remain an active process when the experiment was terminated at 378 days.

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Photomicrographs were prepared by the Armed Forces Institute of Pathology.

[*Illustrations follow*]

Legends for Figures

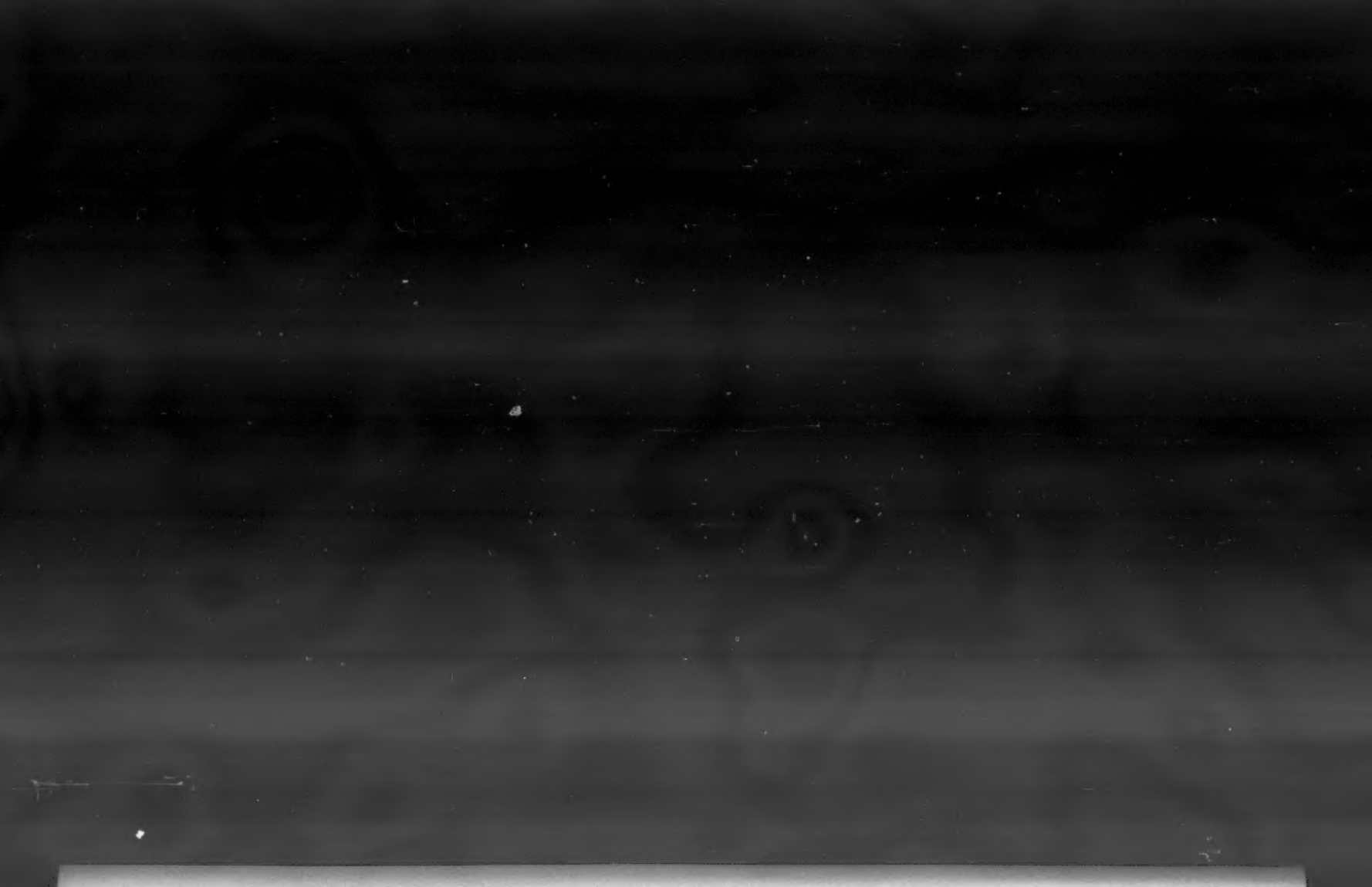
Except where indicated, photomicrographs were prepared from sections stained with the Giemsa stain.

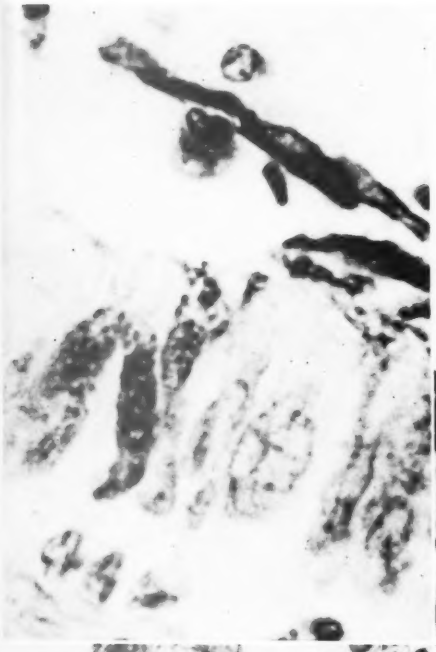
Fig. 1. An arthrospore within a macrophage adjacent to the epithelial surface of a bronchiole, 24 hours after exposure. × 1,330.

Fig. 2. A swollen arthrospore on the epithelial surface of a bronchiole, 24 hours after exposure. × 630.

Fig. 3. A spherule on the surface of a bronchiole. × 440.

Fig. 4. A spherule within an alveolus, 72 hours after exposure. × 440.





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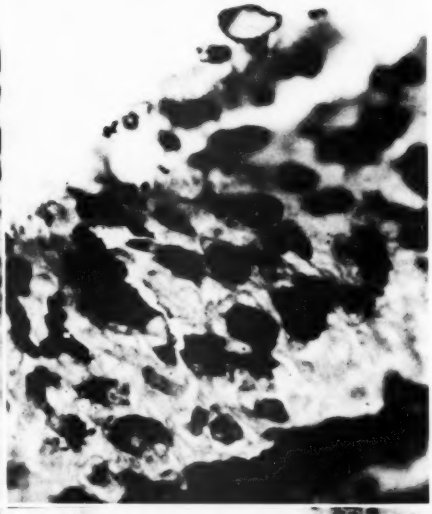

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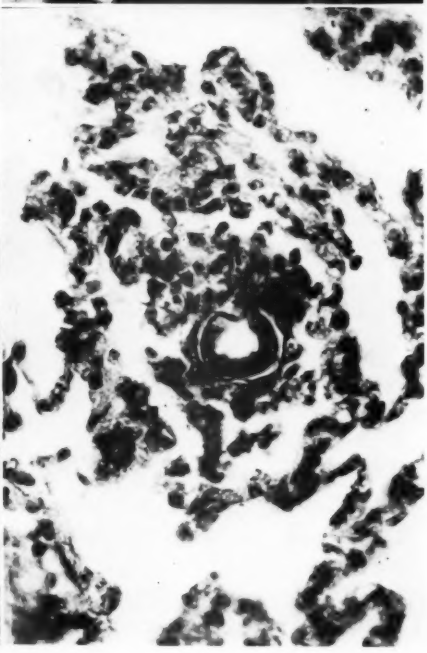

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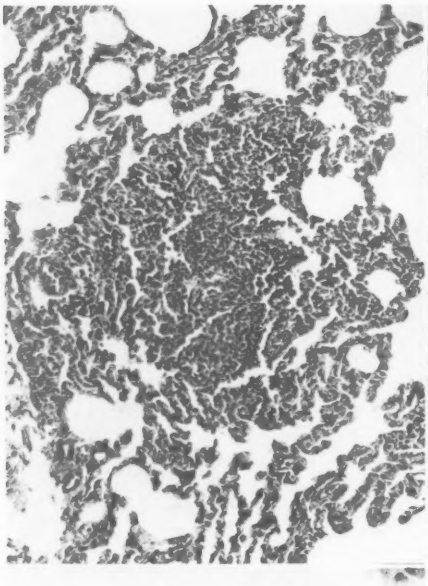

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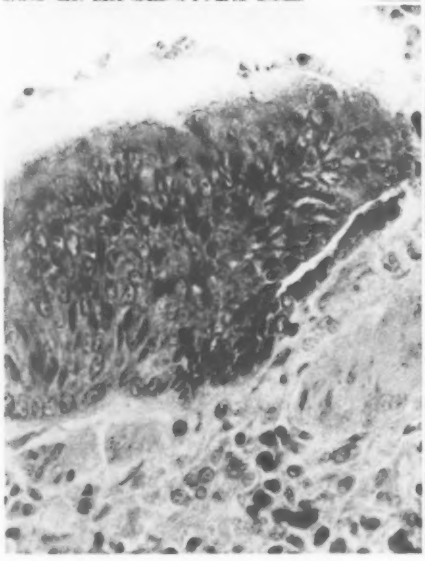

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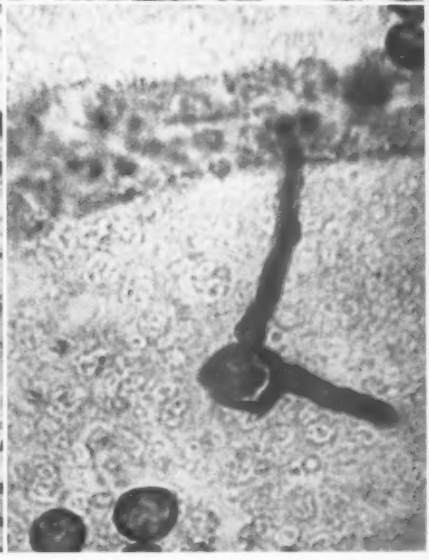

8

FIG. 5. Small, discrete lesion, 5 days after exposure. × 280.

FIG. 6. Newly released endospores in a lesion, which resemble *Histoplasma capsulatum*, 5 days after exposure. Periodic acid-Schiff stain. × 630.

FIG. 7. Low polyp-like mass of bronchial epithelium and adjacent layer of flattened epithelium with large hyperchromatic nuclei, 43 days after exposure. ×440.

FIG. 8. Elongated forms, possibly growing from endospores. × 450.